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# UNWRAPPING THE ROLE OF WRAP53β IN DNA DAMAGE RESPONSE

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# UNWRAPPING THE ROLE OF WRAP53β IN DNA DAMAGE RESPONSE

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my family*

# ABSTRACT

WRAP53β is a WD40 domain protein with multifaceted capabilities in several biological processes in different cellular compartments. This includes transportation of the Survival of motor neuron (SMN) complex, small Cajal body-specific (sca) RNAs and the telomerase enzyme to Cajal bodies, as well as targeting of the E3 ligase RNF8 to DNA double-strand breaks (DSBs).

In **Paper I**, we established WRAP53β as a novel player in the DNA damage response. WRAP53β is rapidly recruited to DNA lesions in an ATM, ATR, H2AX and MDC1 dependent manner, and accumulates at the actual breaks and surrounding regions. WRAP53β interacts with the E3 ubiquitin ligase RNF8 and facilitates binding to MDC1 by simultaneously binding RNF8 and MDC1 via its WD40 domain. This is important for the downstream recruitment of the repair factors 53BP1, BRCA1 and RAD51. Knockdown of WRAP53β impairs DSB repair by both non-homologous end joining (NHEJ) and homologous recombination (HR), causing accumulation of DNA lesions and delayed recovery from G2/M cell cycle arrest.

In **Paper II**, we introduced a sensitive method for analyzing the recruitment of repair factors and formation of protein-protein complexes at DNA lesions. By applying a fluorescence visualization technique called the proximity ligation assay (PLA), we observed accumulation of WRAP53β in close proximity to γH2AX in an ATM and ATR dependent manner, which we also confirmed by co-immunoprecipitation. Moreover, we visualized the interaction between MDC1 and RNF8 via PLA and confirmed that these interactions are dependent on WRAP53β and ATM. Our results also demonstrated that WRAP53β does not interfere with MDC1 phosphorylation by ATM but instead contributes directly to the MDC1 and RNF8 interaction.

In **Paper III**, we studied the role of overexpressed WRAP53β in DSB repair. We have demonstrated that overexpression of WRAP53β disrupts Cajal body formation, and at the same time, enhances the efficiency of DSB repair by HR and NHEJ. Accordingly, γH2AX foci were cleared faster in irradiated cells overexpressing WRAP53β. We also observed increased ubiquitylation of histone H2AX and that this response was attenuated when the responsible enzyme RNF8 was knocked down. WRAP53β overexpressing cancer cells were also resistant to irradiation and several damage-inducing agents. Altogether, this indicates that overexpressed WRAP53β enhances RNF8-mediated ubiquitylation at DNA lesions and thereby increases the repair efficiency and improves cancer cell survival.

In summary, we have identified WRAP53β as an essential player in the DNA damage response (DDR). We could verify its function at damage sites by using the PLA technique and furthermore showed overexpression of this protein enhances DNA repair efficiency.

# LIST OF SCIENTIFIC PAPERS

I. Henriksson S, **Rassoolzadeh H***, Hedström E*, Coucoravas C, Julner A, Goldstein M, Imreh G, Zhivotovsky B, Kastan MB, Helleday T and Farnebo M. The scaffold protein WRAP53β orchestrates the ubiquitin response critical for DNA double-strand break repair. Genes & Development. 2014 Dec 15;28(24):2726-38

II. **Rassoolzadeh H**, Coucoravas C and Farnebo M. The proximity ligation assay reveals that at DNA double-strand breaks WRAP53β associates with γH2AX and controls interaction between RNF8 and MDC1. Nucleus 2015 Sep 3;6(5):417-24

III. **Rassoolzadeh H**, Henriksson S, Hedström E and Farnebo M. Overexpression of the scaffold WD40 protein WRAP53β enhances the repair of and cell survival from DNA double-strand breaks. Manuscript.

*Authors contributed equally

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# 1 LIST OF ABBREVIATIONS

| | |
|---|---|
| 53BP1 | p53 binding protein 1 |
| AP | Apurinic-apyrimidinic |
| ATM | Ataxia-telangiectasia mutated |
| ATR | Ataxia-telangiectasia and Rad3-related protein |
| ATRIP | ATR-interacting protein |
| BARD1 | BRCA1-associated RING protein 1 |
| BIR | Break-induced replication |
| BER | Base-excision repair |
| Bp | Base pairs |
| BRCA1 | Breast cancer 1 |
| BRCC36 | BRCA1/BRCA2-containing complex subunit 36 |
| BRCT | BRCA1 carboxyl-terminus |
| CAB | Cajal body localization box |
| CB | Cajal body |
| CDK | Cyclin-dependent kinase |
| CHD4 | Chromatin helicase DNA-binding protein 4 |
| Chk2 | Checkpoint kinase 2 |
| CTCF | CCCTC-binding factor |
| DC | Dyskeratosis Congenita |
| DDR | DNA damage response |
| dHJ | Double Holliday junction |
| DNA | Deoxyribonucleic acid |
| DNA-PKcs | DNA-dependent protein kinase, catalytic subunit |
| DSB | Double-strand break |
| DSBR | Double strand break repair |
| DUB | Deubiquitylating enzymes |

| | |
|---|---|
| FHA | Forkhead associated domain |
| GAR1 | Glycien arginine rich protein 1 |
| GRR | Glycine-rich region |
| HECT | Homology to E6AP carboxyl-terminus |
| HERC | HECT and the RLD domain containing E3 ubiquitin protein ligase 2 |
| HR | Homologous recombination |
| HSP | Heat shock protein |
| HU | Hydroxurea |
| IR | Ionizing irradiation |
| IRIF | IR-induced foci |
| JMJD2A | Jumonji domain 2A |
| K6-polyUB | Non-canonical lysine-6 linked ubiquitin chains |
| L3MBTL1 | Lethal(3)malignant brain tumor-like 1 |
| LIG3 | Ligase 3 |
| MDC1 | Mediator of the DNA damage checkpoint 1 |
| MIU | Motifs interacting with ubiquitin |
| MMR | Mismatch repair |
| Mre11 | Meiotic recomobination 11 |
| mRNA | Messenger RNA |
| ncRNA | Non-coding RNA |
| NBS1 | Nijmegen breakage syndrome 1 |
| NER | Nucleotide excision repair |
| NHEJ | Non-homologous end joining |
| NOP56 | Nucleolar protein 56 |
| NOP58 | Nucleolar protein 58 |
| NSCLC | Non-small cell lung cancer |

| | |
|---|---|
| NuRD | Nucleosome remodeling and histone deacetylation |
| OTUB1 | OUT domain ubiquitin aldehyde-binding 1 |
| PAR | Poly(ADP-ribose) |
| PARP | Poly(ADP-ribose) polymerase |
| PCD | Programmed cell death |
| PIAS | Protein inhibitor of activated STAT |
| PIKK | Phosphatidylinositol 3-kinase-related kinase |
| PLA | Proximity ligation assay |
| PNKP | Polynucleotide kinase/phosphatase |
| PRR | Proline-rich region |
| PSMD4 | Proteasome 26S subunit non-ATPase 4 |
| PST | Proline-serine-threonine |
| RAP80 | Receptor-associated protein 80 |
| RBR | RNA binding region |
| RBR | Ring between ring |
| RING | Really interesting new gene |
| RNA | Ribonucleic acid |
| RNF8 | Ring finger protein 8 |
| RNP | Ribonucleoprotein |
| scaRNA | Small Cajal body-specific RNA |
| scaRNP | Small Cajal body-specific RNP |
| SDSA | Synthesis-dependent strand annealing |
| SMART | Simple modular architecture research tool |
| SMA | Spinal muscular atrophy |
| SMN | Survival of motor neuron |
| snRNA | Small nuclear RNA |
| snRNP | Spliceosomal small nuclear RNP |

| | |
|---|---|
| snoRNP | Small nucleolar RNP |
| SNP | Single nucleotide polymorphism |
| SSA | Single-strand annealing |
| SSB | Single-strand break |
| ssDNA | Single-stranded DNA |
| STUbL | SUMO-targeted ubiquitin ligase |
| SUMO | Small ubiquitin-like modifier |
| SUV39H2 | Suppressor of variegation 3-9 homologue 2 |
| TCP-1 | T-complex protein 1 |
| TDP2 | Tyrosyl DNA phosphodiesterase 2 |
| TERC | Telomerase RNA component |
| TERT | Telomerease reverse transcriptase |
| Tip60 | 60 KDa TAT-interactive protein |
| TOPBP1 | Topoisomerase binding partner 1 |
| TRiC | TCP-1 ring complex |
| UBC13 | Ubiquitin-conjugating enzyme 13 |
| UDR | Ubiquitylation-dependent recruitment |
| UNRIP | Unr-interacting protein |
| UPS | Ubiquitin-proteasome system |
| USP3 | Ubiquitin-specific proteases 3 |
| USP16 | Ubiquitin-specific proteases 16 |
| UTR | Untranslated region |
| UV | Ultraviolet |
| XLF | XRCC4-like factor |
| WRAP53 | WD40-encoding RNA antisense to p53 |
| XRCC4 | X-ray repair cross-complementing protein 4 |
| WSTF | Williams syndrome transcription factor |

| | |
|---|---|
| XRCC1 | X-ray repair cross-complementing protein 1 |

# 2 INTRODUCTION

## 2.1 THE *WRAP53* GENE

The WD40-encoding RNA antisense to p53 (*WRAP53*) gene is located on chromosome 17p13 just upstream of the *p53* gene on the opposite strand in a head to head fashion and encodes at least 17 splice variants. The gene has three alternative starting exons; 1α, 1β and 1γ (1).

1γ exon is located furthest upstream of the gene and overlaps the first intron of the *p53* gene. However, there is no known function of this transcript. In contrast, transcription from 1α and 1β leads to the production of two independent products with known activities, which gives the *WRAP53* gene a dual function (1). Interestingly, the WRAP53 1α exon overlaps the first exon of p53 in an antisense fashion by up to 227 base pairs (bp) and transcription from this exon produces an antisense RNA, referred to as WRAP53α. Exon 1β is located further downstream, around 930 bp from p53 exon 1 and transcription from this exon produces a WD40 protein referred to as WRAP53β upon translation (other alias; WRAP53, TCAB1, and WDR79) (Figure 1) (1, 2).

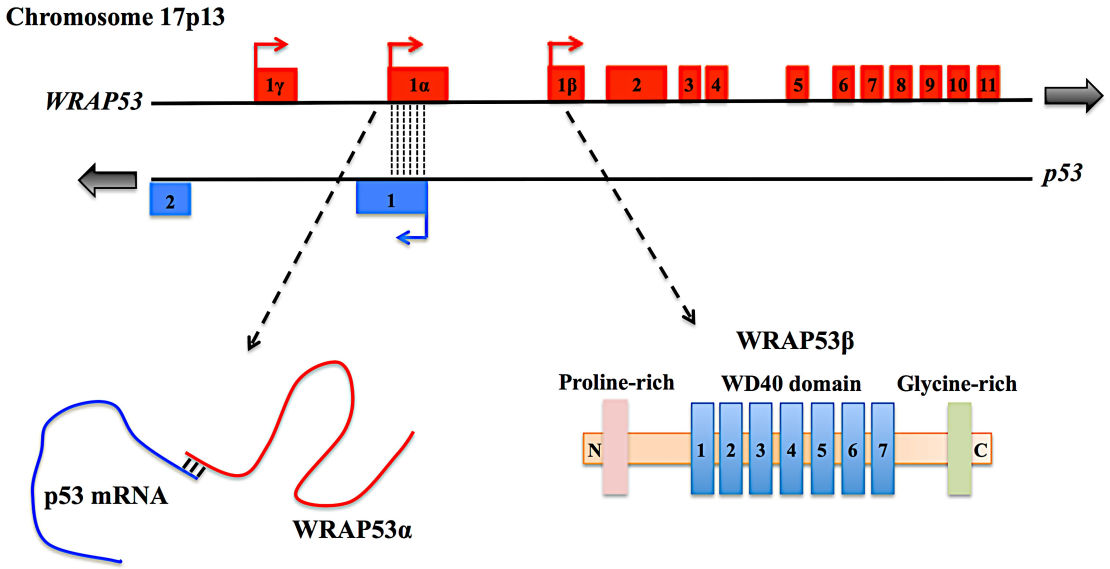


**Figure 1:** Schematic illustration of the *WRAP53* gene with the antisense RNA termed WRAP53α and the WD40 protein termed WRAP53β depicted.

## 2.2 WRAP53α antisense RNA

Sense-antisense gene pairs often consist of a protein-coding gene on the sense strand and a long non-coding RNA (ncRNA) encoding gene on the opposite antisense strand. Antisense transcripts have been shown to contribute to transcriptional control by stabilizing the sense messenger RNA (mRNA) (3).

The 5'untranslated region (UTR) of p53 is an important region for its mRNA stability (4). The WRAP53α transcript has a perfect complementary sequence to the 5'UTR of p53, which enables their interaction. Disruption of this interaction by knocking down WRAP53α or blocking formation of WRAP53α/p53 hybrids leads to decreased p53 RNA levels. Conversely, overexpression of WRAP53α increases p53 expression. Therefore, WRAP53α is an important stabilizer of the p53 mRNA, critical for the function of p53 in the DNA damage response and apoptosis (1).

Recently, the CCCTC-binding factor (CTCF) was identified as a contributing factor in the WRAP53α-mediated p53 stabilization. CTCF is a multifunctional binding factor with several different functions, including transcriptional regulator, insulation (restricting enhancer-promoter interaction) and chromatin organizer. Genome-wide association studies have shown widespread binding sites of CTCF to different promoters, enhancers and intergenic regions through its DNA-binding domain where one of its target sites is the p53 gene (5-7). Moreover, CTCF comprises a RNA binding region (RBR) that binds various transcripts genome-wide. Interestingly, one of them was WRAP53α RNA and direct interaction between CTCF and WRAP53α was important for the regulation of p53 RNA expression (8).

## 2.3 WRAP53β

### 2.3.1 WD40 proteins – domain and functions

WD40 repeat proteins are highly abundant in eukaryotes and in humans there are 349 predicted WD40 domain proteins based on a simple modular architecture research tool (SMART) database (9). The WD40 domain is characterized by the presence of repeated units of 44-60 amino acid residues where each unit ends with a tryptophan (W) and aspartate (D) dipeptide (10). Each repeat contains a four-stranded antiparallel β-sheet that forms a strong hydrogen bond network, which together with the other repeats results in the stable folding of a β-propeller architecture. A WD40 propeller contains four to eight WD40 repeats (Figure 2) (11, 12).

WD40 proteins are involved in many different biological processes such a cell cycle control, apoptosis, chromatin dynamics, gene expression regulation, histone methylation, genome stability, signal transduction, vesicular trafficking and cytoskeletal assembly (13, 14), where they serve as scaffolds for interacting partners. The three-dimensional β-propeller architecture of the WD40 domain enables interaction with partners from different angles via the top region, the bottom region and the circumference of the propeller. This allows formation of large and dynamic multi-protein complexes as well as interaction with DNA and RNA (15).

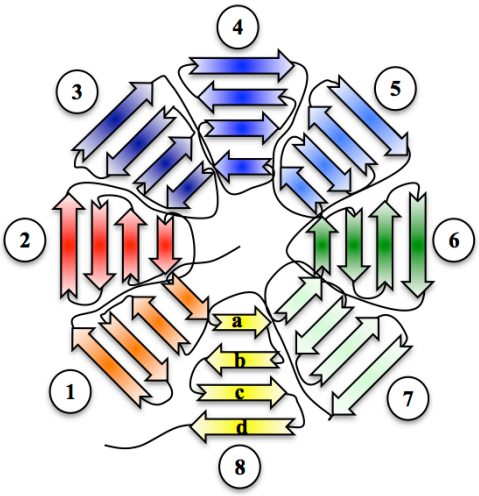


**Figure 2:** Schematic illustration of a β-propeller WD40 domain with eight repeats and four-stranded antiparallel β-sheet named a-b. Each color represents one WD40 repeat.

### 2.3.2 Structure and folding of WRAP53β

Folding of polypeptides into their three-dimensional structure is orchestrated by a highly organized proteostasis network that controls protein biogenesis, conformational maintenance and degradation.

Chaperones conduct protein folding and are divided into different classes, including the heat shock proteins (HSP) 70, HSP90 and chaperonins. HSP70/90 are responsible for the vast majority of chaperone-mediated folding, which folds primarily the "easy" proteins. They mediate folding either as monomers or homodimers by interacting with sections of hydrophobic amino acid on nascent polypeptides, thereby preventing these sections from aggregating until the polypeptides are folded properly.

Chaperonins fold proteins that are difficult to fold. These proteins have a more complex structure and are more sensitive to changes in the general cellular environment and chaperonins keeps the individual polypeptides of these proteins isolated by encapsulating them (16, 17). One such chaperonin is the T-complex protein 1 (TCP-1) ring complex (TRiC). This entity is an eight subunit ATP dependent type II chaperonin complex that functions to stabilize polypeptides with complex topologies in a non-stress inducible environment (Figure 3) (18, 19).

Misfolding of proteins may cause different diseases. Fortunately, processes are available to discard the misfolded proteins by degradation through the ubiquitin-proteasome system (UPS) via the 26S proteasome that requires at least partially unfolded proteins. Degradation of misfolded proteins and folded proteins that are not degradable by the 26S proteasome may also occur via the less preferable autophagy machinery and/or lysosomal/vacuolar

degradation (20-23). Unfortunately, maintaining the proteostasis declines during aging, which leads to accumulation of misfolded proteins, increased deposition of aggregates, cellular toxicity and cell death. These outcomes may in turn lead to degenerative diseases such as Parkinson's, Huntington's and Alzheimer's disease (Figure 3) (20, 24-28).

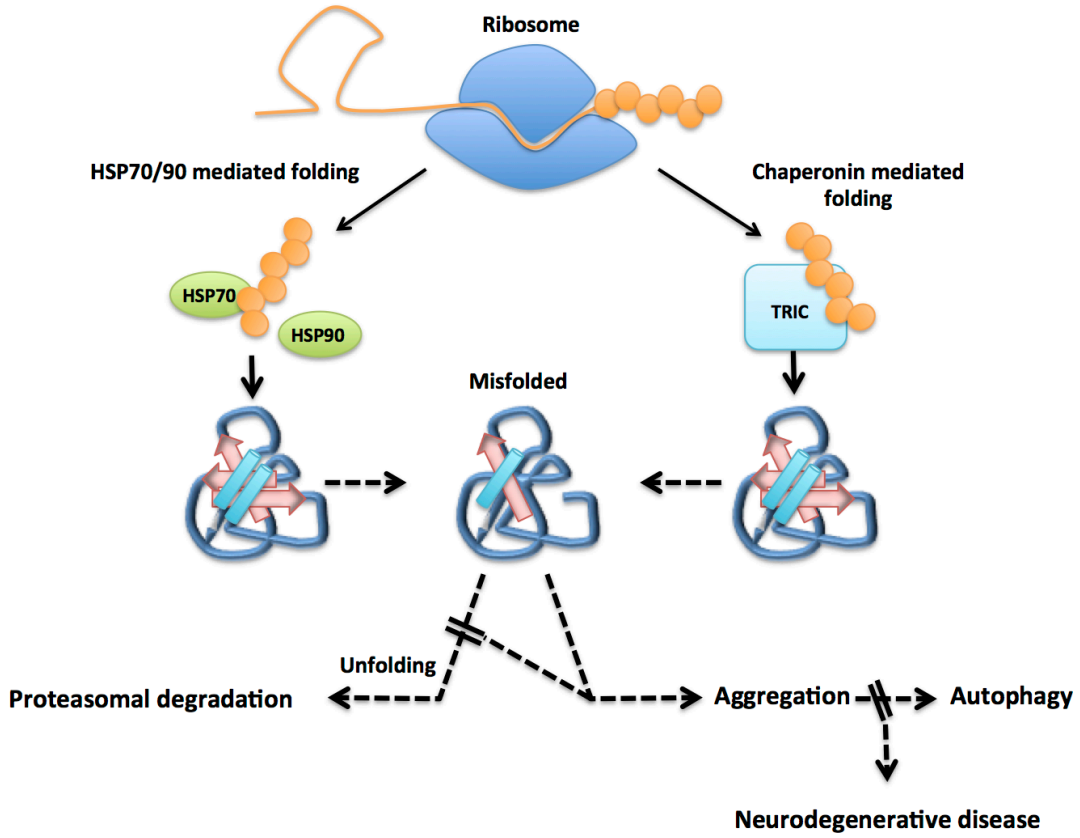


**Figure 3:** Simplified schematic illustration of the proteostasis network. Polypeptides are folded into three-dimensional structures through either HSP70/90 or chaperonin. Misfolded proteins are degraded through either proteasomal degradation or through autophagy.

WD40 proteins are considered to have a complex topology and several WD40 proteins are folded by TRiC (18, 29). WRAP53β is a WD40 protein with seven predicted WD40 repeats a recent report demonstrated that WRAP53β is folded by TRiC (16, 30). Knockdown of any of the eight TRiC subunits resulted in reduced expression and aberrant localization of the WRAP53β protein while knockdown of the HSP70 family members did not have any effect. Loss of TRiC only affected the WRAP53β protein and not its mRNA expression. In addition, TRiC binds the WD40 domain of WRAP53β in the cytoplasm and both the N- and C-termini of WRAP53β are dispensable for this interaction. Still, the C-terminus of WRAP53β is essential for proper folding and release from TRiC. Impaired folding of WRAP53β by TRiC resulted in telomere shortening and was also linked to WRAP53β-associated dyskeratosis congenita, as further described below(16).

In addition to its WD40 domain, WRAP53β contains a N-terminal proline-rich region (PRR) and a C-terminal glycine-rich region (GRR) with unclear functions (Figure 1). PRR is known as a docking site for signaling modules (31-33), while GRR is a known RNA-binding domain involved in RNA recognition and processing (34).

### 2.3.3 Role of WRAP53β in Cajal bodies

WRAP53β is localized in both the cytoplasm and in the nucleus and is enriched in a nuclear organelle known as the Cajal body (CB) (35).

CBs were described for the first time by Santiago Ramón y Cajal in 1903 as small spherical organelles with a size range between 0.2-2μm only visible in the nucleoplasm (36). The number of CBs varies among tissues (1-10 per nucleus), where the number and the size of CBs correlates with the metabolic and proliferative activity of the tissue and being mostly abundant in cells with high splicing and transcription rates (37, 38). Furthermore, CBs are highly dynamic and mobile structures that move to and from nucleoli. As such, they are capable of merging into larger structures and separating into smaller bodies (39). The lack of a phospholipid membrane barrier makes it possible for many factors to move in and out from CBs, which gives them a dynamic structure and accumulation capacity for many factors (40).

CBs are highly concentrated in a variety of factors, including SMN, ribonucleoproteins (RNP) complexes such as spliceosomal small nuclear RNPs (snRNPs), small nucleolar RNPs (snoRNPs), small Cajal body-specific RNPs (scaRNPs), components of the telomerase RNP complex, the p80 protein Coilin, WRAP53β, many ncRNAs from the families of small nuclear RNA (snRNA) and scaRNA. All of these factors are important for the maturation of snRNPs, snoRNPs and the telomerase RNP, as well as for splicing and telomere maintenance (38). It is belied that CBs function as a compartment that increases the efficiency of these processes by concentrating all factors involved. Indeed, cells survive in the absence of CBs, probably because these essential processes can still take place in the nucleoplasm but to a lower efficiency (41-43).

#### *2.3.3.1 Maintenance of Cajal bodies*

Several studies have shown that WRAP53β is highly enriched in CBs in several cell lines and is a vital factor in the maintenance and localization of factors to this organelle (30, 35, 44). Interestingly, cells lacking WRAP53β displays collapsed CBs, are unable to reform and the CB factors coilin and SMN are mislocalized to nucleoli. Loss of all CBs is also observed in cells absent of coilin, while loss of SMN only reduces the number of CBs (35).

CBs are also disrupted by overexpression of high levels of exogenous WRAP53β, which may be due to an adverse effect on the endogenous WRAP53β function by self-association between exogenous and endogenous WRAP53β (35). Similar results have been reported for overexpression of coilin (45). Thus, WRAP53β, together with coilin, maintains the structure and formation of CBs.

#### *2.3.3.2 Localizing the SMN complex to Cajal bodies*

SMN is a 38 kDa protein encoded by the two genes *SMN1* and *SMN2* that plays an important role in the cytoplasmic assembly of splicing snRNPs (46, 47). SMN oligomerizes through its self-association domain and interacts via its Tudor domain with RG (motifs rich in arginine and glycine) boxes of coilin and Sm proteins (48, 49). However, SMN does not assemble snRNPs on its own but function together with at least nine other proteins, including Gemins 2-8, Unr-interacting proteins (UNRIPs), in a complex called the SMN complex (50).

Our group showed that WRAP53β facilitates the import of the SMN complex from the cytoplasm to the nucleus by mediating interaction between SMN and the nuclear pore receptor of Importin-β and furthermore, the localization of the SMN complex to Cajal bodies by mediating interaction between SMN and coilin (35).

#### *2.3.3.3 Localizing scaRNAs to Cajal bodies*

ScaRNA are ncRNAs and this RNA family consists of at least 24 members that are specifically localized in the CBs where they guide the factors responsible for the post-transcriptional modification of snRNA during the maturation process of snRNPs. ScaRNAs are divided into three distinct classes: C/D box, H/ACA box and a mixture of both motifs, which is based on their guide-specificity, sequence element and structural motifs (51). The C/D scaRNAs comprise two sequence motifs called the C (UGAUGA) and D (CUGA) box and guide methylation of snRNA. The H/ACA scaRNA, are comprised of the H (ANANNA) and the ACA motifs, which guides the isomerization of uridine into pseudourine. The C/D scaRNA brings a complex of four proteins: the nucleolar protein 56 (NOP56), NOP58, NHP2L1/15,5K and the methyltransferase fibrillarin, the latter of which is responsible for the 2´-O-methylation of snRNAs. The H/ACA scaRNAs carries a complex of four other proteins: the Glycine arginine rich protein 1 (GAR1), NHP2, NOP10 and the pseudouridine synthase dyskerin. Both C/D and H/ACA scaRNAs direct the associated enzymes to snRNAs via sequence complementary (52, 53). The modified sites on snRNAs are well conserved and distributed in functionally important loci involved in RNA-RNA and RNA-protein

interactions. Thus, modification of snRNAs alters their structure and enhances interactions with RNA and proteins important for their function in pre-mRNA splicing (Figure 4) (54).

The H/ACA and the mixture H/ACA-C/D scaRNAs contain a Cajal body localization box (CAB box, a tetranucleotide sequence ugAG) that targets them to CBs (51, 55). WRAP53β interacts directly with this CAB box and thereby recruits scaRNA to CBs. Depletion of WRAP53β or mutation in the scaRNA CAB box that obstructs their interaction leads to mislocalization of the scaRNA to the nucleoli (44). The C/D scaRNA, on the other hand, does not contain the CAB box, and therefore it remains unclear how C/D scaRNA are recruited to the CBs. Although it has been shown that WRAP53β interacts with C/D scaRNA, but 20-fold less efficiently compared to H/ACA scaRNA (44, 56). Yet it has been shown recently that the main interaction partner with the C/D scaRNA is coilin, which is involved in the processing of certain C/D scaRNAs. However, another research group have shown that C/D scaRNAs instead contain a GU- or UG-dinucleotide-rich repeat sequences, called the G.U/U.G wobble stem and is predicted to form a terminal stem-loop of the RNA apical hairpin. This novel Cajal body localization element is critical for the interaction of C/D box scaRNPs with WRAP53β, and their subsequent localization to Cajal bodies (figure 4) (56-59).

In addition to binding the CAB motif of H/ACA scaRNAs, WRAP53β also interacts with CAB box-containing AluACA RNAs (60). Full-length Alu elements are about 300 nt long with two monomer units (left and right arms) that are present in more than one million copies in the human genome in a non identical sequence (60, 61). The AluACA RNAs are processed from intron-encoded Alu RNAs and mature AluACA RNAs are structurally similar to H/ACA scaRNAs and associate with the H/ACA core proteins: NOP10, NHP2, GAR1, and dyskerin. However, H/ACA scaRNA that only carries one CAB box on either of the 3’ or 5’ hairpins, the 3’ hairpin of Alu ACA RNAs carry two CAB boxes, which interacts with WRAP53β and accumulate in the nucleoplasm. . Moreover, unlike H/ACA scaRNAs, which accumulate in Cajal Bodies, AluACA RNAs accumulate in the nucleoplasm. It is not known if the differentially arranged CAB boxes contribute to the different subcellular localization of the H/ACA scaRNPs and AluACA RNPs and what the main function of the AluACA RNPs is (60).

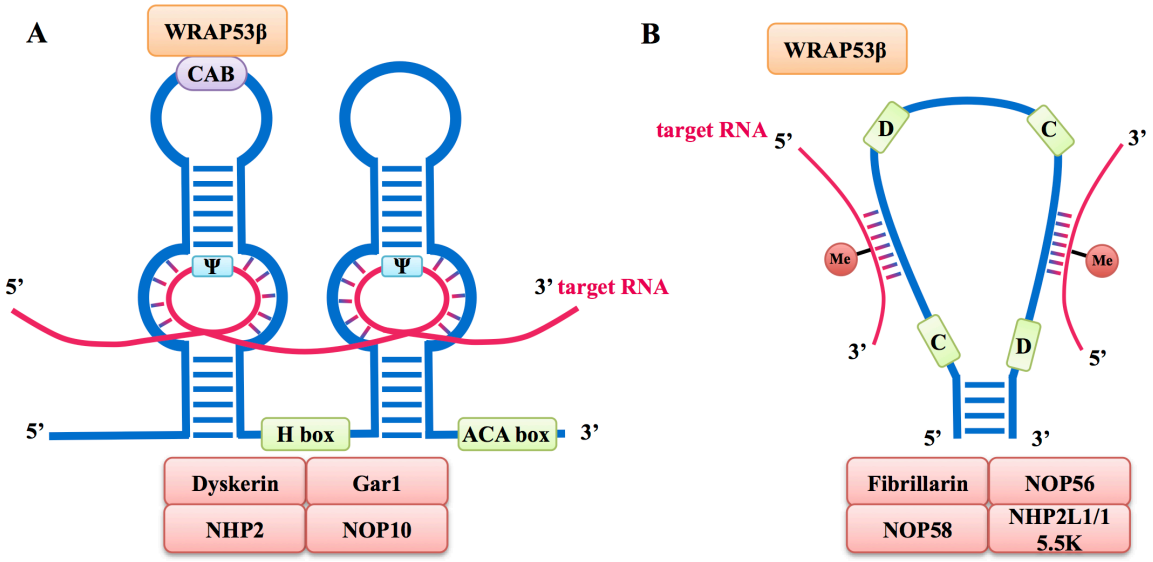


**Figure 4:** An illustration of a H/ACA box (A) and C/D box (B) scaRNA. **A.** Targeted snRNA (red) interacts with the H/ACA scaRNA and becomes pseudouridylated (Ψ) by dyskerin. WRAP53β interacts with the CAB motif and facilitates transportation of scaRNAs to CBs. **B.** Targeted snRNA (red) interacts to complementary sequence of C/D scaRNA located in between the C and D box and becomes 2´-O-methylated by fibrillarin.

### *2.3.3.4 Localizing the telomerase complex to Cajal bodies and telomeres*

Telomeres consist of short repetitive TTAGGG hexanucleotide units that span over 2-15 kb at the end of chromosomes and are ended with a G-rich 3' single-stranded region of about 100-200 bp (62). The overhanging 3' single-stranded region invades the double-stranded region by creating a t-loop structure that acts as a closed structure to prevent chromosomal end fusion (63). In addition, the six subunit based shelterin complex mediates the t-loop structure by capping the double-stranded and single-stranded telomeric DNA (62). Telomeres lose about 100-200 bp after each full cell cycle, which gives primary human cells a lifespan of 60-80 population doublings before they become senescent and die by apoptosis to prevent genomic instability (64, 65). Unfortunately, cancer cells have the ability to counteract telomere shortening and evade senescence by reactivating the telomere enzymes (66).

The telomere enzyme synthesize the addition of the telomeric repeats on telomeres and consist of the enzyme telomerase reverse transcriptase (TERT) and the telomerase RNA component (TERC).

TERC is a 451 nt long H/ACA containing scaRNA that acts as a template for DNA synthesis (67, 68). As mentioned earlier, the H/ACA box provides the assembly of the associated proteins such as dyskerin and the binding of dyskerin to TERC is important for the stability of TERC and the biogenesis of the telomerase RNP. However, there is no evidence that dyskerin uses TERC as a guide for pseudouridylation of target RNAs (69, 70). TERC also

contains the CAB box that allows interaction with WRAP53β and thereby enables targeting of TERC (in association with the active telomerase enzyme) to CBs and further on to telomeres by WRAP53β, which is essential for telomere elongation. The assembly of TERC into an active telomerase enzyme is independent of WRAP53β and most likely occurs prior to binding to WRAP53β. Knockdown of WRAP53β or mutations in the CAB box of TERC does not affect the levels of TERC but disrupts targeting of TERC and the associated complex to CBs and telomeres, which leads to progressive telomere shortening. Furthermore, WRAP53β associates with all telomerase factors including TERT and dyskerin. The association with TERT is believed to be mediated through the interaction to TERC since the association between TERT and WRAP53β was lost upon RNase A treatment. However, it is believed that the interaction with dyskerin is based on protein-protein interaction due to its insensitivity to the RNase A treatment (Figure 5) (30, 71, 72).

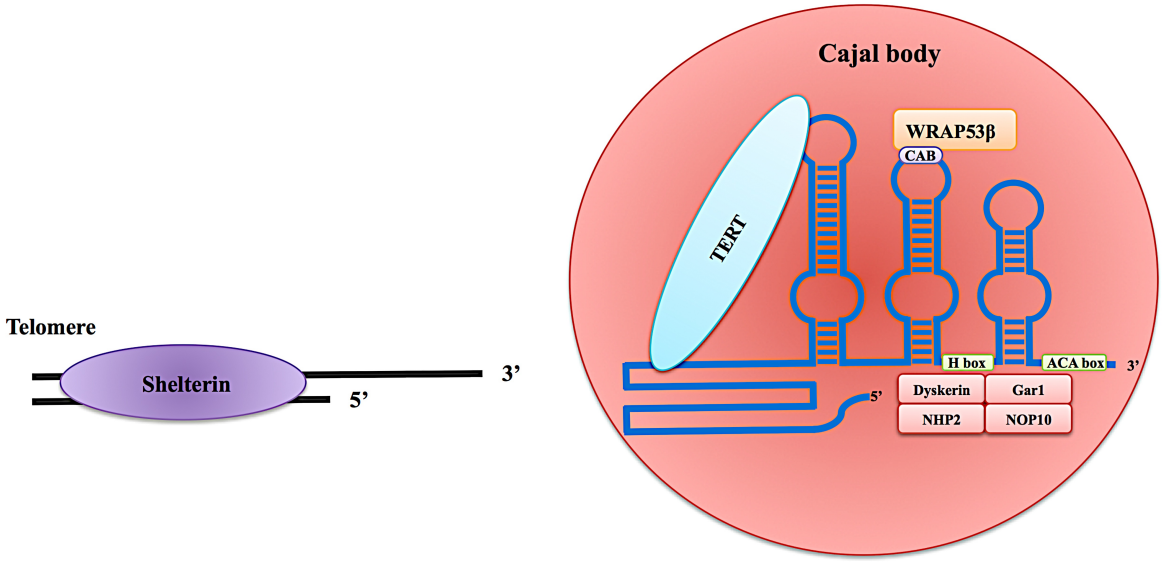


**Figure 5:** WRAP53β targets TERC, Dyskerin and TERT to CBs by interacting with the CAB box of TERC and subsequently transport telomerase to telomeres.

### 2.3.4 Role of WRAP53β in inherited diseases

#### *2.3.4.1 Dyskeratosis congenita*

Telomere biology disorders include several different diseases. These overlapping conditions are cause by inherited mutations in factors involved in different aspects of telomere maintenance. Dyskeratosis congenita (DC) is the most common telomere biology disorder, characterized by bone marrow failure, premature aging, cancer predisposition, skin pigmentation, nail dystrophy and oral premalignant leukoplakia. The most frequent tumors in DC patients are myelodysplastic syndrome, acute myelogenous leukemia and the solid tumors squamous cell carcinoma of the head/neck and anogenital cancer (73, 74). DC

patients also display very short telomeres and impaired stem cell function due to defective and insufficient telomere maintenance. As many as nine genes have been identified mutated in patients with DC where the different genes are inherited through X-linked, autosomal recessive or autosomal dominant patterns (Figure 6) (73, 75, 76).

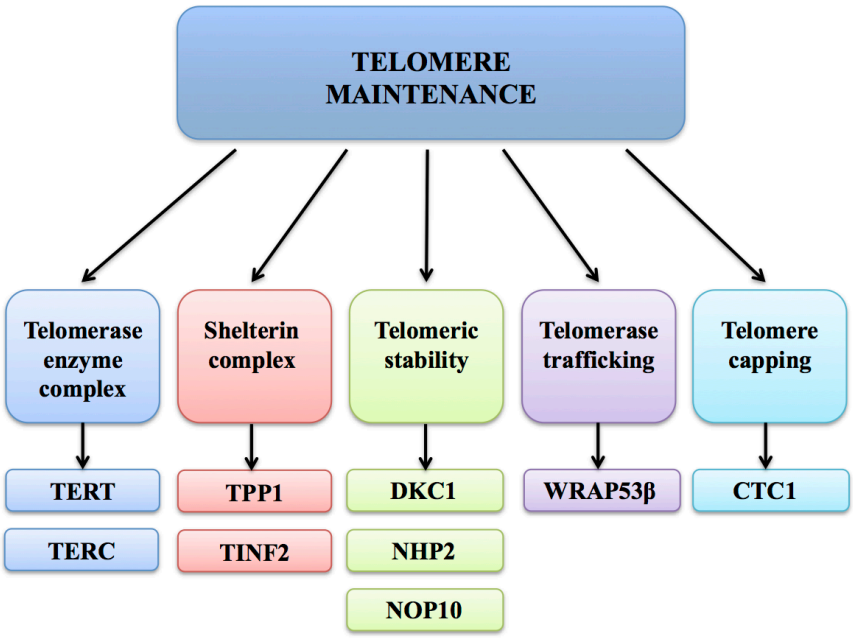


**Figure 6:** A scheme of proteins with genetic abnormalities associated with DC and their function in telomere maintenance.

Germline mutations in WRAP53β cause DC and so far four missense mutations in WRAP53β have been identified in two patients, inherited in an autosomal recessive fashion. The first patient displayed mutations in exon 2 and 8 (F164L and R398W, respectively) of WRAP53β and the second patient had mutations in exon 7 and 9 (H376Y and G435R, respectively). This caused severely reduced protein expression of WRAP53β, especially in the nuclear compartment, while the mRNA levels of WRAP53β were unaffected, indicating that the protein is downregulated post-translationally in these patients. Since the mutated residues all locate in the highly conserved WD40 domain of WRAP53β they are believed to impair the function of this domain. Indeed, mutations in WRAP53β prevent TRiC-mediated folding resulting in reduced stability of WRAP53β protein and its mislocalization within the cell, i.e. inability of WRAP53β to enter the nucleus. As a consequence, TERT is not localized to CBs (instead accumulate in nucleoli) and telomere elongation is disrupted (16, 77).

### *2.3.4.2 Spinal muscular atrophy*

Spinal muscular atrophy (SMA) is a neurodegenerative disorder where patients have progressive degeneration of spinal cord anterior horn α-motor neurons, muscle atrophy and

motor impairment due to homozygous deletion or mutation in the *SMN1* gene. This condition is the leading genetic cause of infant mortality worldwide and is further divided into four different classifications (SMA I-IV) that are based on age of onset and disease severity. SMA type I is the most common and severe variant with signs of the disease appearing soon after birth (49, 78, 79).

The human genome contains two genes encoding the SMN protein, *SMN1* and *SMN2*, due to a large inverted duplication located in chromosome 5q. These two genes are almost identical and only differ only in a critical single nucleotide in exon 7 (C to T transition) of *SMN2* that disrupts an exon splicing enhancer. This leads to a potential redirection towards alternative splicing, where exon 7 is excluded from SMN2-derived mRNA transcripts, resulting in a truncated SMN protein that is rapidly degraded (80-84). Although a small portion of the mRNAs produced from *SMN2* includes exon 7 and give rise to the full-length SMN protein, the *SMN2* gene cannot compensate for the loss of SMN protein caused by deletions or mutations in *SMN1*. Interestingly, multiple copies of the *SMN2* gene may exist due to the instability of chromosome 5q caused by duplication and the amount of these copies correlate with disease severity, where higher copy number correlates with milder disease variants (85). Patients with five *SMN2* copies have been found to completely compensate for homozygous *SMN1* deletion thus preventing SMA development (86).

Why the motor neurons in the spinal cord are selectively killed in SMA patients is unclear. It has been suggested that motor neurons have higher energy requirements and express SMN at the levels of demand while other tissues express more than necessary (87, 88). Moreover, SMN is essential for the assembly of the heptameric Sm ring on snRNAs and thus plays a key role in the splicesomal snRNP biogenesis pathway, which could be of particular importance for motor neurons. Accordingly, both animal and cell culture models have displayed a correlation between reduced snRNP assembly and disease severity in motor neuron degeneration. It has also been shown that the targeting of SMN and coilin to CBs is defective in SMA type I motor neurons (89-92). Our data indicate that WRAP53β may be involved in the latter scenario, since the WRAP53β-SMN interaction is disrupted in fibroblasts from SMA type I patients, which could contribute to the failure of SMN and coilin in localizing to CBs (35).

### 2.3.5 Role of WRAP53β in cancer

WRAP53β is overexpressed in several different cancer cell lines of different origin and its overexpression promotes carcinogenic transformation of NIH3T3 cells. Moreover, cancer cell

lines become apoptotic through the mitochondrial pathway when WRAP53β is knocked down while normal cells are unaffected (93). This indicates that WRAP53β has oncogenic properties when overexpressed and that cancer cells get addicted to WRAP53β (oncogenic addiction). Furthermore, WRAP53β is overexpressed in primary esophageal squamous cell carcinoma, non-small cell lung cancer (NSCLC), primary nasopharyngeal carcinoma and rectal cancer (94-97). Knockdown of WRAP53β in NSCLC and head and neck cancer cell lines reduced size of the tumors formed when these were grafted into mice (95, 96).

As mentioned earlier, WRAP53β is an important factor in telomere elongation, which could partially explain its oncogenic properties. After all, constant telomerase activation is what immortalizes 90% of all human cancers (98). On the other hand, mutations in both alleles of WRAP53β cause DC, which indicates that WRAP53β acts as a tumor suppressor rather than an oncogene. In line with this, reduced expression of WRAP53β in epithelial ovarian, breast and head and neck cancer correlates with poor patient survival (99, 100). Furthermore, single nucleotide polymorphisms (SNPs) in the *WRAP53* gene increase the risk of breast and ovarian cancer development (101, 102).

Together this indicates that WRAP53β may have different functions in cancer depending on cancer types or stage of tumor development, which influences whether WRAP53β should act as a tumor suppressor or as an oncogene. Moreover, the subcellular localization of WRAP53β is of importance for its cancer susceptibility properties, since loss of nuclear but not cytoplasmic WRAP53β is correlated with reduced survival and radioresistance in head and neck cancer patients (103).

## 2.4 THE DNA DAMAGE RESPONSE

About 50,000 DNA lesions occur daily where the majority are base modifications (104, 105). However, about ten of these lesions are DSBs, which are considered to be the most cytotoxic form of DNA damage and require a more sophisticated and complex reparation machinery. Efficient and proper repair of all different types of damage is required for maintaining the genomic integrity and an ineffective repair may lead to mutations, cell death and severe diseases such as cancer, neurodegeneration, immunodeficiency and radiosensitivity (106). The majority of DNA lesions arise from endogenous activities, including hydrolytic reactions, non-enzymatic methylations, byproducts produced from oxidative respiration or through redox-cycling events. Some also arise due to physiological processes such as mismatches during DNA replication or due to unsuccessful topoisomerase I and II activities (107).

DNA damage also arises by exogenous agent, including ultraviolet (UV) light. Even though the ozone layer removes most of the dangerous UV rays, residual UV-A and UV-B from extensive sun exposure can induce approximately 100,000 lesions per exposed cell and hour of sunlight. Additionally, exposure to ionizing irradiation (IR) from for example radon gas, tobacco smoke and overcooked meat induce DNA damage that is connected to different cancer types (108, 109).

To counteract the threats posed by DNA damage, cells have developed several repair mechanism that activates a signaling cascade collectively called the DNA damage response (DDR). The DDR serves to maintain genomic integrity by detecting DNA lesions, signaling their presence and promoting repair (110, 111). However, the DDR facilitates different repair pathways depending on the type of lesion acquired and the majority of lesions are repaired by catalytic events involving multiple proteins. The mismatch repair (MMR) is an important repair system during DNA replication and recombination that corrects mismatches of bases by triggering a single-strand incision and then resynthesizing by DNA polymerase (112). Meanwhile, the base-excision repair (BER) pathway corrects damage of a single nitrogenous base that is recognized and removed by a DNA glycosylase enzyme to produce an apurinic-apyrimidinic (AP) site. The site is further processed by an AP endonuclease and other factors to prepare the strand to be resynthesized by a DNA polymerase (113). Helix-distorting base lesions caused by UV light is repaired by nucleotide excision repair (NER), where the repair involves the removal of about 22-30 oligonucleotides creating a stretch of single-stranded DNA (ssDNA), which is then refilled by a DNA polymerase (114). DSBs are repaired by two repair systems called homologous recombination (HR) and non-homologous end joining (NHEJ) including several subtypes as further described below.

### 2.4.1 DNA double-strand breaks

DSBs arise when the phosphodiester bond is broken simultaneously in close proximity on opposite DNA strands. This could be extremely toxic for the cell if not repaired since it for example may lead to genome rearrangement. A DSB requires a more complex repair system, since most probably some nucleotides are lost due to the damage, while single-strand DNA breaks (SSBs) still have a physically undamaged strand that can be used as template to restore the damage strand (115).

DSBs may occur naturally during meiotic recombination and during V(D)J recombination, the latter being an important maturation step of the immune system. However, DSBs arise also at sites of collapse of the DNA replication forks or due to topoisomerase II cleavage

(116, 117). Several exogenous sources may also induce DSB such as topoisomerase inhibitors, DNA damage inducing agents, radiomimetic drugs and IR (118).

Upon DSB induction, several factors are involved in sensing the region where the damage has occurred and activates the signal transducers that amplify and transduce signals to downstream effectors. Eventually, mediators are activated by posttranslational modification by different sensors and transducers to coordinate the spatio-termporal regulation of all different factors involved in the DDR. This is done by promoting activation, maintaining recruitment and controlling the association of repair factors with damaged DNA. The transducers activate different downstream effectors depending on the response required such as cell cycle arrest effectors, DNA repair effectors (e.g., NHEJ or HR) programmed cell death (PCD) or apoptosis effectors (119).

### 2.4.2 DNA damage signaling

Since there are different pathways available (e.g., NEHJ and HR) for DSB repair, the appropriate sensor needs to be activated to promote further signaling and subsequently promote the required repair pathway for the most efficient repair. However, it is yet not directly known how a particular DSB promotes the recruitment of one over another DNA damage-sensing factor. Even so, it is believed that several factors may be attributed to the choice of the sensor such as the cell cycle where the HR pathway can only be utilized during the S/G2 cell cycle phase. This is due to the requirement of a sister chromatid as a template sequence whereas the NHEJ pathway repairs throughout the cell cycle (120).

The poly(ADP-ribose) polymerase (PARP) proteins PARP1 and PARP2 can sense both single- and double-stranded DNA damage, whereas PARP3 predominately responds to DSBs (121). The roles of PARPs in SSB involve activation of their enzymatic activities and the synthesization of poly (ADP)-ribose (PAR) chains on themselves and other proteins. In this manner, PARPs promote the recruitment of the X-ray repair cross-complementing protein 1 (XRCC1) and the DNA ligase 3 (LIG3) (122-124). Although their importance in DSBs is less studied and highly debated, recent data has suggested that PARP1 is involved in HR repair pathways of hydroxurea (HU)-mediated collapsed replication forks by recruiting the meiotic recombination 11 (Mre11) nuclease and initiating end resection activity (125, 126). On the other hand, PARP3 drives repair towards the NHEJ repair pathway by preventing excessive end-resection mediated by Mre11 and repair by HR (121).

The Ku heterodimer is comprised of Ku70 and 80 and acts as a sensor for the NHEJ pathway by interacting with free DNA ends. It is believed that the sensing capability of Ku in NHEJ is

based on its high affinity for free DNA ends. The high DNA binding affinity is independent of DNA sequence and allows for a rapid binding (about 5 seconds), which is important for stabilizing the DNA ends and for recruiting downstream NHEJ factors (127, 128).

The MRN complex is the major sensor of DNA DSBs and is implicated also in DNA replication fork restart, telomere maintenance, signaling to the cell cycle checkpoints and meiosis (129, 130). The complex is comprised of three proteins: Mre11, RAD50 and the Nijmegen breakage syndrome 1 (NBS1). The Mre11/RAD50 complex interacts with the damaged DNA ends as a heterotetramer through Mre11. The DNA ends are brought in close proximity by RAD50 globular domains from which anti-parallel coiled coil structures extend to Zn-hook domains. This process facilitates RAD50 dimerization (131). NBS1 on the other hand recruits and activates the ataxia-telangiectasia mutated (ATM) kinase, which mediates downstream signaling (132, 133).

#### *2.4.2.1 Phosphorylation-mediated signaling*

Phosphorylation mediated signaling is coordinated by the apical phosphatidylinositol 3-kinase-related kinases (PIKKs): ATM, ataxia-telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). These three kinases phosphorylate serine or threonine residues followed by a glutamine residue (S/TQ). Several hundred potential substrates have been identified for ATM and ATR (134, 135), while DNA-PKcs itself is the only relevant DNA-PK substrate identified so far *in vivo*, but *in vitro* this latter enzyme phosphorylates several DDR factors (136-138). All three kinases mediate the phosphorylation of the histone variant H2AX on serine 139 (referred to as γH2AX), which is one of the earliest events in the DDR cascade (139, 140). ATR is most important for H2AX phosphorylation during replication stress or following DNA end resection (141). ATR is recruited together with its interaction partner ATR-interacting protein (ATRIP) and is then activated by the topoisomerase binding partner 1 (TOPBP1) (142, 143). It has also been shown that the MRN complex mediates ATR signaling where RAD50 is phosphorylated by ATR even though the MRN complex primary function is to recruit ATM to DSBs. Phosphorylated RAD50 is an important factor for DNA replication restart by promoting loading of cohesin at the site of damage (131). While ATR primarily promotes γH2AX formation in replication stress, ATM is the major player in phosphorylating H2AX in response to DSBs. Yet DNA-PKcs function redundantly and overlap in γH2AX formation (144, 145).

ATM is present in its inactive form as a non-covalently linked dimer (146). ATM becomes activated upon DSB formation by dimer disassociation into monomers, which leads to activation of its enzymatic activity and autophosphorylation at serine 1981. Eventually ATM is recruited to DSB by the MRN complex (147). However, besides autophosphorylation and the MRN complex, the acetyltransferase 60 KDa TAT-interactive protein (Tip60) is required for maximal ATM activation where Tip60 is recruited to DSB in a complex with ATM by interaction with H3K9me3 and H3K36me3 leading to acetylation of ATM on lysine 3016, histone H4 and histone H2A (148-151).

The human nucleosome consists of a histone octameric core comprised of four different histone types: H2A, H2B, H3 and H4. The histone types constantly undergo different posttranslational modifications such as phosphorylation, ubiquitination, sumolytion, PARylation, methylation and acetylation (152). The dynamic combination of histone posttranslational modification influences gene expression, DDR signaling and repair (153, 154). H2AX is a member of the H2A family and accounts for about 10% of total H2A molecules in human fibroblasts and varies between 2-25% in other cell types. In addition, H2AX has a longer C-terminal tail with a conserved SQ phosphorylation motif as compared to H2A. The discovery of the SQ site as responsive to DNA damage-induced phosphorylation was a crucial step in establishing H2AX as an important player in the DDR (155).

As mentioned earlier, γH2AX formation requires phosphorylation of H2AX at serine 139 by the PIKKs but H2AX needs to be methylated before being phosphorylated. The suppressor of variegation 3-9 homologue 2 (SUV39H2) is a SET containing methyltransferase that methylates H3K9 and has been shown to dimethylate H2AX on lysine 134. Loss of methylated H2AX has revealed substantially lower levels of γH2AX formation and expression and also leads to reduced affinity between the PIKKs and H2AX. Therefore, ATM and ATR at least need to bind to H2AX K134 to be able to mediate the phosphorylation at S139 for γH2AX formation (156).

Formation of γH2AX at DSBs reaches its maximum within minutes post-damage and can be detected up to 2 mega bases away from the DSB. This is important for facilitating focus formation, retention of repair proteins and chromatin changes at DSBs (157-159). Even though there is evidence indicating that γH2AX is an important factor in DNA damage signaling in both HR and NHEJ pathways, it is not essential for the initial localization of DDR factors such as the p53 binding protein 1 (53BP1), breast cancer 1 (BRCA1) or the MRN complex. Additionally, only mild defects in DNA damage checkpoint controls and DNA repair processes were observed in H2AX deficient cells. Therefore, it is believed that

γH2AX is important for optimizing repair efficiency by concentrating proteins in the vicinity of DSBs (160-162).

Interestingly, H2AX may also be phosphorylated on tyrosine 142 by the Williams syndrome transcription factor (WSTF) remodeling factor kinase, which seems to be important in chromatin remodeling (163, 164). γH2AX is eventually dephosphorylated when the lesion has been repaired and several phosphatases such as PP1, PP2A, PP4 and WIP1 are involved in this dephosphorylation. However, a portion of the phosphorylated H2AX is also evicted from the nucleosome by histone exchange (165).

Mediator of the DNA damage checkpoint 1 (MDC1) is a large protein with five domains: forkhead associated domain (FHA), SDT, TQXF, proline-serine-threonine (PST) and BRCA1 carboxyl-terminus (BRCT), which enable it to interact with several DDR factors. The tandem BRCT domain mediates phosphorylation-dependent interaction and is located in the C-terminus of MDC1. The BRCT domain of MDC1 directly binds γH2AX, which enables MDC1 to act as an adaptor protein that interacts with other proteins through its many other domains (166, 167). The PST domain is the least conserved one and little is known about its function. This domain is not required for MDC1 accumulation at DSBs, its function in mediating accumulation of downstream DDR factors, nor for activation of HR or NHEJ (168). The FHA domain in the N-terminus of MDC1 mediates the interaction with ATM, the checkpoint kinase 2 (Chk2) and RAD51 (169). ATM phosphorylates the TQXF motif of MDC1, which facilitates interaction of MDC1 with the E3 ubiquitin ligase ring finger protein 8 (RNF8). Chk2 phosphorylates the SDT domain, which facilitates interaction with NBS1, important for the MRN complex retention at DBSs. Interaction with ATM, NBS1 and γH2AX enables MDC1 to directly or indirectly mediate the recruitment and retention of activated ATM and the continuous phosphorylation of H2AX located more distant to the damage site (170-173).

MDC1 residence time at the damage site is controlled by small ubiquitin-like modifier (SUMO)ylation mediated by protein inhibitor of activated STAT (PIAS)1/4. SUMOylated MDC1 is recognized by the SUMO-targeted ubiquitin ligase (STUbL) RNF4 that ubiquitylates MDC1 and this modification is recognized by proteasome 26S subunit non-ATPase 4 (PSMD4) and leads to proteasomal degradation (174, 175).

The PIKKs also phosphorylate effector molecules that regulate cellular processes. These include transcription, senescence, apoptosis and delayed cell cycle progression. Chk1 and Chk2 are two well characterized substrates of ATR and ATM, respectively (176). Chk1 is

important for the activation and maintenance of the G2/M checkpoint whereas Chk2 is mainly important for the G1/S checkpoint (177). Both entities act by reducing the cyclin-dependent kinase (CDK) activity to mainly slow down or arrest cell-cycle transition at the G1/S, intra S and G2/M cell-cycle checkpoint. This is believed to be important in avoiding a non-repaired damaged DNA to pass through the checkpoints and also in increasing the time available for the repair before replication and mitosis take place. The principal substrate for Chk1 is CDC25A, which leads to $SCF^{\beta TRCP}$-mediated degradation and activation of intra S and G2/M checkpoints (178). At the same time, p53 is the critical substrate of Chk2, which promoting stabilization of p53 and subsequent apoptosis when the amount of DSBs is more than the system is capable of repairing (179).

#### *2.4.2.2 Ubiquitin-mediated signaling*

Ubiquitin is a 76 amino acid residues small protein, encoded by four genes in the human genome (UBC, UBB, UBA52 and UBA80), which were first described as being fused to ribosomal proteins or as linear polyubiquitin chains (180-182). Full-length ubiquitin is a precursor peptide that needs to be processed through cleavage to expose the carboxyl-terminal di-glycine motif that is covalently conjugated through its carboxyl-terminal to the ε-amino group of the targeted proteins lysine. The conjugation of ubiquitin requires an enzymatic process, which is divided to three steps: E1-(activating), E2-(conjugating) and E3-(ligase) enzymes (183, 184). The number of enzymes involved in the conjugation varies greatly among the "E" enzymes. There are eight known E1s of which only two are known to be specific for ubiquitin, 35 E2s and a prediction of more than 1000 E3s (185-187). The E3s are divided into three families: really interesting new gene (RING), homology to E6AP carboxyl-terminus (HECT) and ring between ring (RBR) (187). The RING E3 ligases do not contain any catalytic activity and do not interact with ubiquitin directly but rather mediate the ubiquitylation by interacting with the E2 and transferring ubiquitin from E2 to the targeted substrate. This is done by positioning the ubiquitin into a favorable position for conjugation (188, 189). On the other hand, HECT and RBR physically transfer the ubiquitin from E2 to its active site cysteine and then to the substrate (190). Some substrates are mono-ubiquitylated on a single lysine residue while others are multi-mono-ubiquitylated, which have been shown to be important in regulating lysosomal degradation of proteins and mediating protein-protein interactions (191, 192). Ubiquitin contains seven lysine residues; K6, K11, K27, K29, K33, K48 and K63. These residues enable the formation of different types of ubiquitin chains and poly-ubiquitylation of substrates, where the type of ubiquitin linkages is largely determined by the pairing of specific E2-E3s (190). The majority of the

lysine-residue-mediated ubiquitylation labels protein for proteasome-mediated degradation. However, K63-linked ubiquitin chains instead mediate protein-protein interactions that are particularly important for the DDR (193, 194).

Ubiquitylation has been shown to regulate almost all DNA repair pathways, especially in early signaling following DSBs where BRCA1, 53BP1 and RAD51 are recruited to damaged sites in an ubiquitin-mediated manner. K63-linked chains are highly enriched at DNA lesions, and the E3 ligase RNF8 is the critical link between phosphorylation and ubiquitylation events in the DDR (195-197). RNF8 contains both a RING domain, which is important for E2 interaction, and a FHA domain that binds phospho-threonine residues, thus facilitating interaction with substrates phosphorylated by the PIKKs. The FHA domain of RNF8 mediates its interaction with MDC1 phosphorylated at its TQXF domain, as previously mentioned (195-197). Moreover, WRAP53β mediates the RNF8-MDC1 interaction by bringing RNF8 to MDC1 (as discussed in paper I). RNF8 interacts with two other proteins that are important for mediating ubiquitylation at DSBs. The E2 ligase ubiquitin-conjugating enzyme 13 (UBC13) interacts with RNF8s RING domain, while ATM-phosphorylated HECT and the RLD domain containing E3 ubiquitin protein ligase 2 (HERC2), another E3 ligase, interacts with the FHA domain of RNF8. HERC2 is believed to stabilize the interaction between RNF8 and UBC13 and maintain RNF8 levels at DNA lesions (198). RNF8 is not the only E3 ligase important in the ubiquitin-mediated recruitment of factors to DSBs. The E3 ligase RNF168 is also critical for repair of DSBs and recruited to DNA breaks in a RNF8 ubiquitylation-dependent manner. It was originally believed that RNF8 induced monoubiquitylation of histone H2A and that this in turn was needed for RNF168 recruitment to break sites (199). However, a recent study identified the H1-type linker histones as the critical substrate of RNF8. H1 linker histones contain many lysine residues and are ubiquitylated under basal conditions. A dramatic increase in K63-linked ubiquitin chains on linker histone H1 has been detected upon damage induction was shown to be dependent on RNF8 and UBC13. The K63-linked ubiquitylation on H1 linker histones is targeted by RNF168, which contains ubiquitin-binding domains known as motifs interacting with ubiquitin (MIU). This mediates its binding to K63-linked conjugates on H1 linker histones. From here, RNF168 in turn catalyzes the formation of K27-linked ubiquitin chains on lysine 13 and 15 of histone H2A and H2AX, which is important for the recruitment of 53BP1, receptor-associated protein 80 (RAP80) and BRCA1 (200-203).

Interestingly, RNF8 can also interact with the E2 ubiquitin-conjugating enzyme UBCH8, which promote K48-linked polyubuiquitylation of several substrates such as Ku80, the

demethylase jumonji domain 2A (JMJD2A) and the polycomb protein lethal(3)malignant brain tumor-like 1 (L3MBTL1) where the two later proteins binds with and masks the H4K20m2 (204-206). The ubiquitin-selective chaperone/segregase VCP/p97 facilitate ubiquitin-dependent extraction of chromatin-associated proteins such as L3MBTL1 and expose histone H4K20me2, which is important for 53BP1 recruitment (206).

The ubiquitin-mediated signaling response is attenuated or turned off by the action of deubiquitylating enzymes (DUBs) and approximately 100 DUBs are encoded by the human genome (207, 208). Several DUBs function to limit RNF8 and RNF168-mediated ubiquitylation. The ubiquitin-specific proteases 3 and 16 (USP3 and USP16) both catalyze the disassembly of RNF8 and RNF168-generated ubiquitin chains (209, 210). The OUT domain ubiquitin aldehyde-binding 1 (OTUB1) inhibits UBC13 by a direct interaction and consequently limits the RNF8 and RNF168 ubiquitylation (211). The BRCA1/BRCA2-containing complex subunit 36 (BRCC36), on the other hand, specifically catalyzes the removal of the K63-linked ubiquitin chains mediated by RNF8 and UBC13 (212). It is also noteworthy that an E3 ubiquitin ligase paralogous to RNF168 called RNF169 acts as a negative regulatory of RNF168-catalyzed ubiquitylation products by competing with 53BP1 and BRCA1 for binding the ubiquitin chains eventually limiting the magnitude of their recruitment to DNA lesions (213).

53BP1 is a 1972 amino acid large protein that contains BRCT repeats, a tandem Tudor domain and 28 amino-terminal S/T-Q site which is phosphorylated mainly by ATM (214). As mentioned earlier, 53BP1 targets H4K20me2 that has previously been exposed in a RNF8-mediated fashion and this interaction is dependent on the tandem Tudor domain of 53BP1. Albeit the interaction to H4K20me2 is necessary for 53BP1 recruitment, it is not sufficient for its focal accumulation at DNA lesions, suggesting an additional binding site of 53BP1 (215). As mentioned earlier, 53BP1 interacts with RNF168 ubiquitin chains and more specifically is highly selective for H2AK15ub through its ubiquitylation-dependent recruitment (UDR) motif located adjacent to the tandem Tudor domain (216). Mutation in the UDR motif impaired 53BP1 focus formation and abolished the interaction with H2AK15ub but not to H4K20me2, which indicates that the binding to H4K20me2 is RNF168 independent. This finding thereby confirms the RNF8-UBCH8 mediated H4K20me2 exposure for 53BP1 (205, 206, 216). Interestingly, 53BP1 must be present at least as a dimer, and it binds to mononucleosomes containing both H2AK15ub and H4K20me2 marks. The BRCT repeats are thought to be dispensable, but recently, a research group used quantitative chemical proteomics to identify 53BP1 BRCT repeats to be binding γH2AX. They detected

that the interactions of 53BP1 with H4K20me2 and H2AK15ub are in general very weak bindings. Therefore, 53BP1's localization to damaged sites relies upon multiple interactions where no single epitope provides sufficient interaction energy for stable association. However, it is believed that the interaction of 53BP1 with γH2AX occurs in a specific context such as DNA lesions in late repairing heterochromatin regions (217, 218).

BRCA1 is a 1863 amino acid large protein with three regions: a RING domain in the N-terminus (a large unstructured region), a coiled-coil domain and tandem BRCT repeats in the C-terminus (219). These three different regions enable the protein to create three binding complexes: BRCA1-A, BRCA1-B and BRCA1-C, where each complex implicates BRCA1 in different multiple cellular functions such as transcription, cell cycle checkpoint activation and DNA repair, most importantly in the HR pathway (Figure 7) (220, 221). The BRCA1 RING domain interacts with the BRCA1-associated RING protein 1 (BARD1), forming a heterodimer, which is important for their mutual stability and this complex is present in all BRCA1 sub-complexes (222-224). The heterodimer can direct both mono- and polyubiquitylation depending on the interacting E2 conjugating enzyme (225). Moreover, they catalyze the formation of non-canonical lysine-6 linked ubiquitin chains (K6-polyUb), which serves as a signaling mechanism for protein complex assembly and/or protein stabilization. These chains are also important for the accumulation of BRCA1 itself and the end resection factor CtIP at DNA lesions (226-228). In the BRCA1-A complex, BRCA1 interacts directly with a protein termed Abraxas through its BRCT repeats in a phosphorylation-dependent manner, which also facilitates binding to RAP80, another BRCA1-A complex component. RAP80 recognizes and interacts with K63-polyubiquitylated chains, mediating the recruitment of BRCA1 to the DNA lesions for repair and also causing inhibition of end resection (229-231). Moreover, the complex consists of several other proteins related to the DUB proteins that provide deubiquitylation important for the regulation of protein dynamics at DNA lesions (Figure 7) (232). In the BRCA1-B complex, BRCA1 binds to Bach1 that is phosphorylated by CDK on S990 in a cell cycle dependent manner. This complex formation is required for S-phase activation during stalled or collapsed replication forks (233-235). Bach1 interacts with TopBP1 that is required for replication-induced checkpoints and also for the extension of single-stranded DNA and RPA loading following replication stress (Figure 7) (236). The BRCA1-C complex is required for DNA end resection and entails CtIP that is phosphorylated at S327 by CDKs in a cell-cycle dependent manner during S and G2 phase. The phosphorylated S327 site is required for binding to the BRCT domain of BRCA1. CtIP also brings the MRN complex to the BRCA1-C complex by interacting with the NBS1 subunit of the MRN complex. It thereby stimulates

the nuclease activity of the MRN complex in order to promote DNA end resection for HR-mediated DSB repair (Figure 7) (234, 237). As mentioned above, all BRCA1 complexes (e.g., A-C) are formed through the BRCT repeats of BRCA1. On the other hand, the coiled coil domain of BRCA1 is an important binding site for the WD40 partner and localizer of BRCA2 (PALB2), which acts as a bridge mediator between BRCA1 and BRCA2 (238-241). BRCA2 in turn interacts with RAD51 and promotes its loading to the resected ssDNA for efficient HR repair (Figure 7) (242).

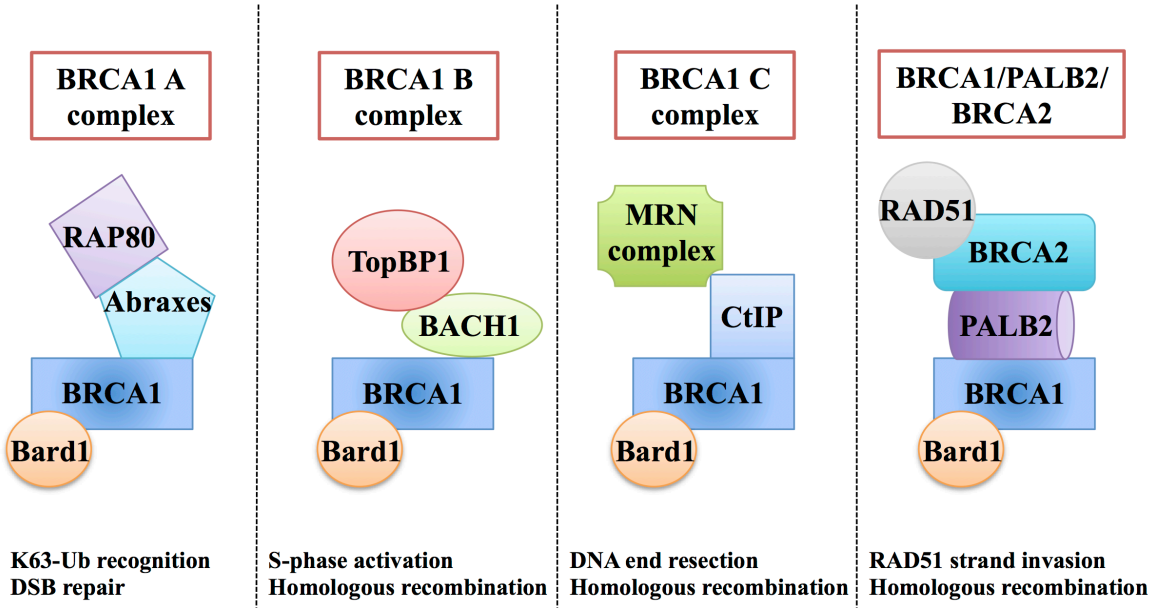


**Figure 7:** An illustration of the different BRCA1 complexes with their interaction partners and function.

Even though, RAD51 is recruited through the BRCA1-C complex, it is still dependent on RNF8 ubiquitylation where RAD51 recruitment has been shown to be impaired upon RNF8 knockdown while unaffected or modestly affected upon RNF168 knockdown and also in RIDDLE syndrome cells with mutated RNF168, respectively (243-245). Additionally, RAD51 recruitment to DNA lesions is RNF8 dependent upon replication. Thus, the recruitment is possibly dependent on K48-linked ubiquitin chains mediated by RNF8 and not through the K63-linked ubiquitin chains mediated by RNF8/Ubc13/RNF168 (204, 246, 247). However, the full mechanism in RNF8 dependent RAD51 recruitment is yet not fully understood. Nonetheless, RAD51 replaces RPA at resected ssDNA in a mechanism dependent on RNF8- and RNF4-mediated recruitment of the proteasomal component PSMD4 and through the E3 ligase RNF18, which interacts with RNF8-generated ubiquitin chains and thereby mediates the recruitment of its binding partner RAD51C, a RAD51 paralog that is necessary for RAD51 foci formation. It has also been suggested that RAD51 is recruited only in the absence of 53BP1 and BRCA1 (175, 248, 249). A summarized schematic scheme of the damage response is illustrated in figure 8.

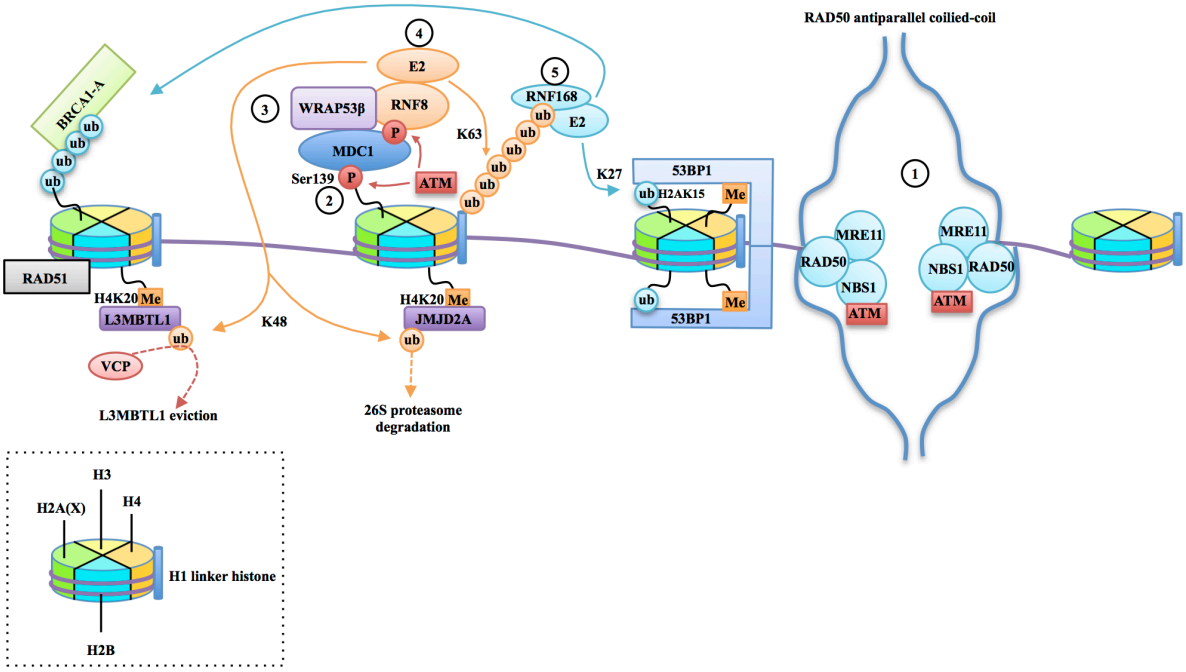


**Figure 8:** A schematic illustration of the DDR. 1) The MRN complex is recruited to the DNA ends, brining ATM with them. 2) ATM phosphorylates H2AX, which interacts with MDC1. 3) WRAP53β recruits RNF8 to MDC1. 4) RNF8 ubiquitylates, through its E2 ligase, H1-linker histone, L3MBTL1 and JMJD2A, thus promoting the degradation of L3MBTL1 and JMJD2A leading to exposure of H4K20me2 thereby creating the docking site for 53BP1. In addition, RAD51 is also recruited. 5) Ubiquitylated H1-linker histone is recognized by RNF168 that further ubiquitylates H2A and H2AX facilitating the recruitment of 53BP1 and BRCA1-A complex.

### 2.4.3 Repair of DNA double-strand breaks

#### *2.4.3.1 Homologous recombination*

HR repairs DSBs through several sub-pathways. Additionally, HR function in the repair of collapsed replication forks and also in maintaining the chromosome ends by promoting telomere recombination (115).

HR-mediated repair is divided into three stages: pre-synapsis, synapsis and post-synapsis. In the pre-synapsis, DNA ends are processed 5’ to 3’ in order to create a 3’ ssDNA overhang, which is initially bound by RPA and then replaced by RAD51 (250). The processing is mediated by four nucleases (the MRN complex, Exo 1, Dna2, and CtIP) and the helicase BLM, where the MRN complex together with CtIP initiates resection and degrade a part of the broken 5’DNA. The DNA is further processed more extensively by Dna2, Exo 1 and BLM (251). RPA bound to the ssDNA restricts RAD51 assembly to DNA, and therefore, RPA becomes displaced through two different classes of mediators in human cells. The first class of mediators is comprised of the RAD51 paralogs: RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3. The second class is mediated through BRCA2 (252, 253). These

factors are all important for presynaptic RAD51 filament formation and stability, however the precise mechanism of action of the mediator proteins is poorly understood.

During synapsis, the RAD51-ssDNA filament performs a homology search by invading an intact DNA molecule, also generating a D-loop where the invading stand primes DNA synthesis. The RAD54 motor protein is required for RAD51 filament stabilization and D-loop enhancement but is especially important for dissociating RAD51 from heteroduplex DNA and enabling the transition from DNA strand invasion to DNA synthesis (254). Finally, in the post-synaptic stage, four different sub-pathways of HR are distinguished to repair DNA lesions.

The double strand break repair (DSBR) sub-pathway creates a so-called double Holliday junction (dHJ) by generating four-stranded DNA structures that is dissolved in two different ways. The dHJ is cleaved into crossover or non-crossover products by several structure specific endonucleases such as: ERCC1-XPF, MUS81-EME1, GEN1 and SLX1-SLX4 (255-257). The non-crossover product keeps its parental configuration, and the two dHJ may migrate towards each other through branch migration. This item is then dissolved by the topoisomerase complex BLM-TOPOIIIα-RMI1/RMI2. The crossover product, on the other hand, produces an exchange of flanking genetic materials with the duplex DNA (258, 259).

In synthesis-dependent strand annealing (SDSA), only non-crossover products are produced by dissolving the D-loop where the invading strand is disengaged after DNA synthesis and annealed with the second end. However, the process may require multiple rounds of invasion, synthesis and disengagement. Break-induced replication (BIR) repairs DSBs that have only one free end by assembling the D-loop into a fully established replication fork to copy the entire distal fragment (260). Conversely, the single-strand annealing (SSA) is a RAD51-independent pathway where DSBs with two ends are flanked by repeated sequence. The repair is instead dependent on RAD52 and RPA by annealing to homologous ssDNA sequences during 5’ end resection and then ERCC1-XPF processes the remaining 3’ flap overhangs (Figure 9) (261).

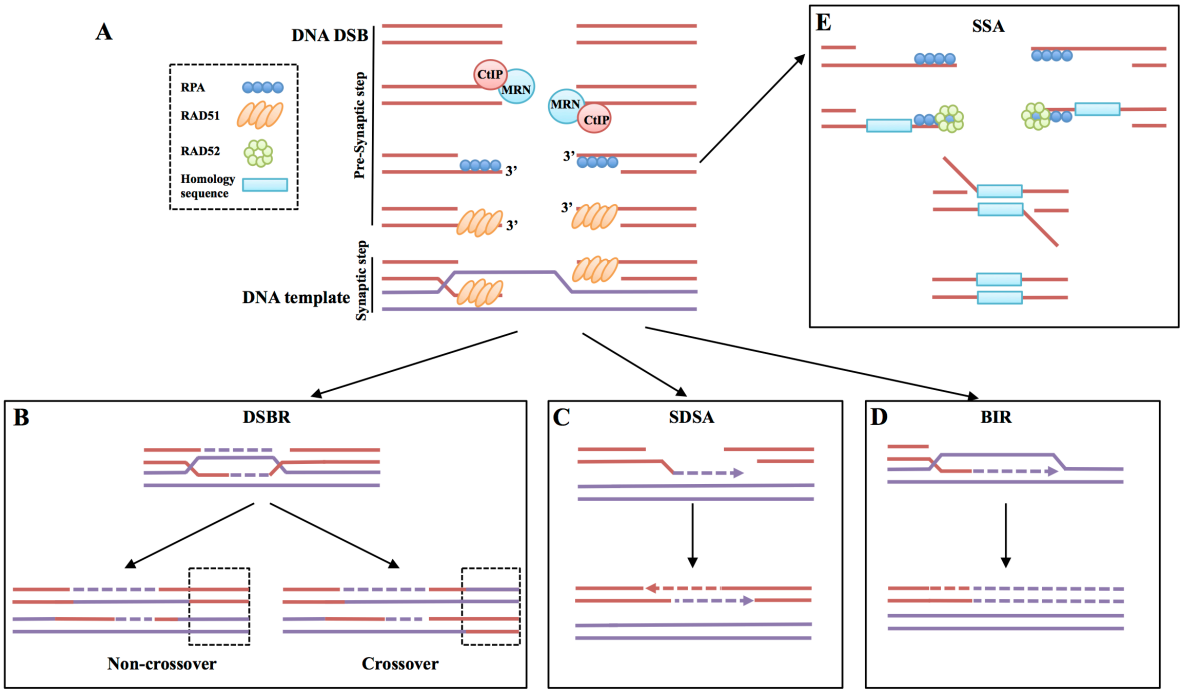


**Figure 9**: Illustration of the Homologous recombination repair pathway. A) The initial steps of HR repair, red represents damaged DNA and blue the template DNA. B) DSBR, the products of non-crossover and crossover are marked in dashed box. C) SDSA. D) BIR. E) SSA.

### *2.4.3.2 Non-homologous end joining*

The NHEJ repair pathway is characterized by rejoining broken DNA ends without using a template, which enables this pathway to be active throughout the cell cycle and therefore is the predominant repair pathway for DSBs in humans (262). This repair pathway is considered to be error prone since it does not utilize a template for restoring any DNA sequence that has been lost upon damage. However, if the lesion is broken with blunt ends where no end processing is required, the DNA sequence might be restored. Conversely, in the absence of clean breaks and or in the presence of base and sugar damage, end processing may be required, which may led to mutagenic events due to loss or gain of genetic sequences (263). NHEJ is divided into five stages: 1) recognition of breaks by Ku, 2) synaptic end bridging, 3) DNA end processing, 4) ligation of breaks and 5) Ku removal from repaired DNA (128).

As mentioned earlier, Ku is the sensor for recognizing DSBs intended in NHEJ repair. Ku is highly abundant with about 500,000 molecules in each human cell with a very strong affinity to DNA ends, recruited within 5s post damage (127). Ku is enriched via its central ring domain where it acts as a scaffold for the entire NHEJ complex. It has been unclear how many Ku molecules are recruited to DSBs. However, recent development in visualizing Ku foci revealed that 2 Ku molecules are recruited to a DSB, one at each end (264). Once attached to the DNA, DNA-PKcs are recruited to lesions and directly interacts with Ku80

through both the N and C terminals of DNA-PKcs (265). Interaction then initiates an active complex, in which Ku starts to translocate and allows DNA-PKcs to be placed at the tip of the end. This allows for synaptic joining of the two ends and stabilization of the complex (266). Additionally, activated DNA-PKcs leads to 15 auto-phosphorylation events and also the phosphorylation of several other NHEJ factors including Ku70/80 *in vitro* (267, 268).

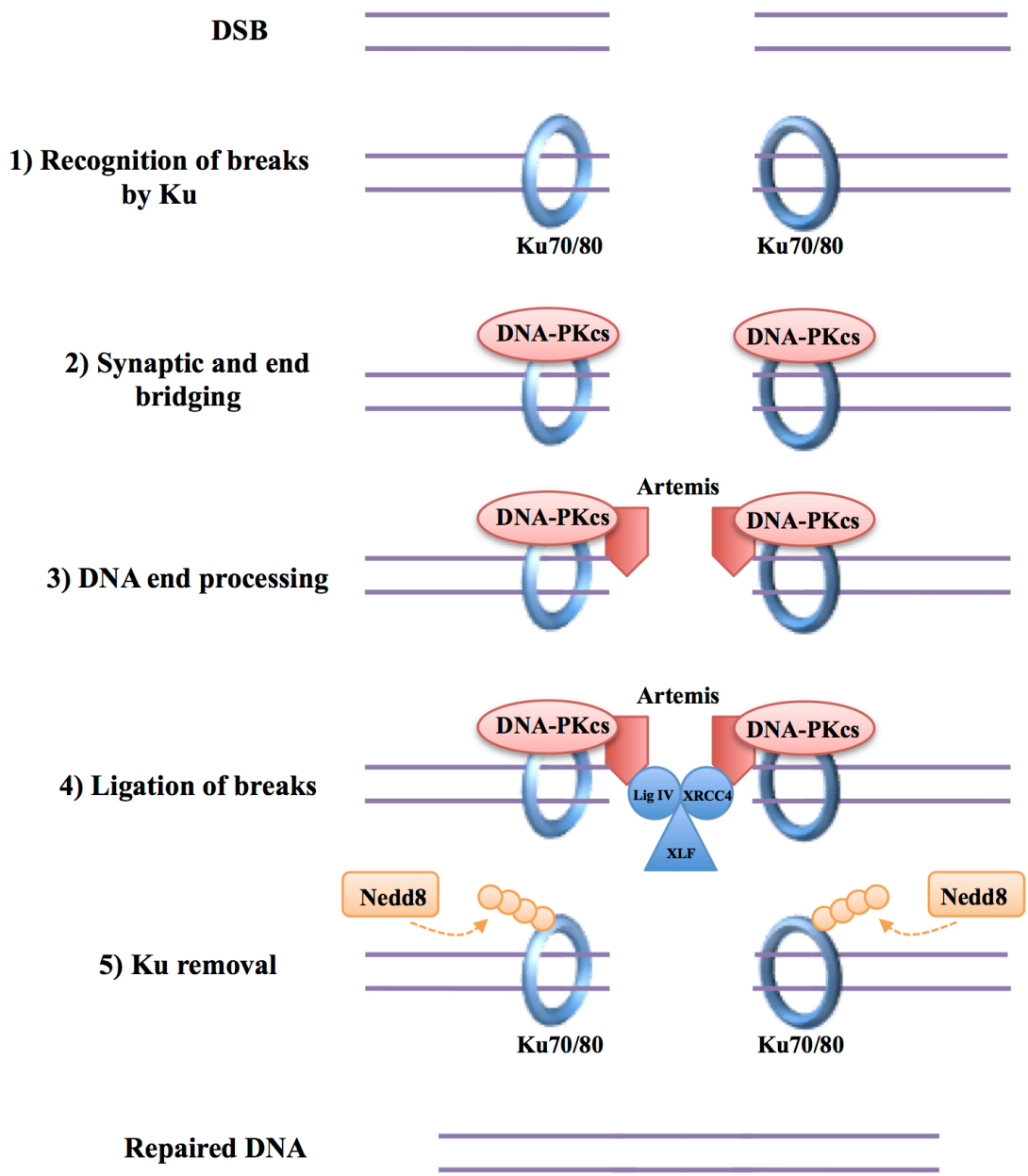


**Figure 10:** Schematic illustration of non-homologous end-joining repair pathway.

Between the break recognition by Ku and the final ligation of the break, there is considerable flexibility in factors involved in the repair, which depends on the type of damage. For example, IR induces several different kinds of damage and also produces non-ligatable 3'-phosphate groups, 3'-phosphoglycolates or 5'-hydroxyl groups. Therefore, several factors are required to process the ends, which involve kinases and phosphatases (polynucleotide

kinase/phosphatase (PNKP)), nucleases (Werner, Mre11, Artemis, ExoI), polymerases (DNA polymerases m and I), helicases (RECQ1) and phosphodiesterases (tyrosyl-DNA phosphodiesterase 1) (128, 136). Meanwhile, topoisomerase II-induced damage leads to physical incorporation of topoisomerase II to the 5'terminus, which requires the specific end-processing enzyme tyrosyl DNA phosphodiesterase 2 (TDP2) by hydrolyzing the topoisomerase II interaction (269, 270). Following end processing, the ends are fused together with help of ligation factors such as DNA ligase IV, X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor (XLF). These elements are subsequently recruited to Ku, forming a heterodimer complex where XRCC4 interacts directly with the Ku70 (271-274). When damage is repaired, Ku needs to be removed since it encircles the DNA and is literally trapped. Several possible mechanisms in Ku removal have been proposed. It has been suggested that K48-ubiquitin-linked chains on Ku mediated by RNF8 promote Ku degradation and RNF8 knockdown leads to increased Ku80 retention at breaks and decreases NHEJ efficiency (204). However, recent studies have implied that it is not RNF8 but instead NEDD8 that ubiquitylate Ku (figure 10) (275).

#### *2.4.3.3 53BP1 and BRCA1 regulation of repair pathway choice*

53BP1 and BRCA1 have shown to be essential for the selection of a repair pathway. It has been observed in BRCA1-deficient cells that 53BP1 inhibits HR by blocking the resection of DSBs, which leads to an aberrant repair by NHEJ. Fascinatingly, the loss of 53BP1 in the BRCA1-deficient cells rescues the disease phenotypes associated with BRCA1 deficiency and restores the RAD51 foci and HR repair (276, 277). The 53BP1 NHEJ promotion is strictly dependent on its interaction with RIF1 in a DNA damage-dependent manner (278). The 53BP1-RIF1 accumulation at DSBs prevents the BRCA1-CtIP recruitment in G1 thereby inhibiting end resection. Therefore, BRCA1-CtIP (BRCA1-C) recruitment is restricted to the S/G2 phase of the cell cycle where they in turn inhibit RIF1 recruitment by promoting resection of DSB ends. Even though 53BP1 accumulates in DSB-induced foci during G1, S and G2 phase, super-resolution microscopy analysis has shown that 53BP1 and RIF1 have been evicted from the focal core during S phase in a BRCA1-dependent manner (279-281).

### 2.4.4 Detection and visualization of DNA double-strand breaks

#### *2.4.4.1 IR-induced foci*

Genotoxic agents such as topoisomerase inhibitors, $H_2O_2$, radiomimetic drugs and IR induce DNA damage that promotes the accumulation of many factors at the site of damage leading to the formation of cytological discernable foci (282-284). Such foci can be detected by

indirect immunofluorescence with antibodies against the protein of interest or in real time by live imaging with fluorescently tagged proteins. Factors forming IR-induced foci (IRIF) normally overlap with γH2AX foci indicating that they form around the damage site. The numbers of foci increase in a dose-dependent manner and it has been estimated that around 20-40 foci are formed per gray of irradiation in human cells and corresponds to approximately 20-40 breaks. Additionally, changes in size and the number of foci throughout the repair time can be visualized (285).

#### *2.4.4.2 Laser micro-irradiation*

The development of laser micro-irradiation (LMI) enabled the study of factors that do not form IRIF and also the study of spatiotemporal accumulation of repair factors at DNA breaks. There are several types of LMI, each using different energy sources to induce damage such as alpha particles, energetic-heavy ions, UV-A laser, two-photon laser, YAG laser and confocal laser. The UV-A laser is the preferred choice, since the dose range is very close to the damage induced by IR (286). LMI is based on a microscope system equipped with a UV-A laser with a wavelength between 337-390 nm. Cells are pre-sensitized to the UV-A light by low levels of either halogenated thymidine analogs (BrdU, IdU) and/or DNA intercalating dyes (Hoechst 33258), which upon UV-A exposure, results in a photochemical reaction that generates DSBs (287).

#### *2.4.4.3 Proximity ligation assay*

Proximity ligation assay (PLA) is a method to detect individual proteins or protein-protein interactions in fixed cells with amplified signals (288). The method is performed in 4 steps: 1) targeting your protein or proteins of interest using conventional antibodies, 2) targeting the primary antibodies using secondary antibodies tagged with oligonucleotide probes, 3) if the antibodies are in close proximity of each other, the oligonucleotides will hybridize with two connector probes and upon ligation form a circular DNA molecule that can be amplified via rolling circle amplification, and 4) the ssDNA molecule produced by rolling circle amplification can be labeled with fluorescently complementary oligonucleotides, which can be detectable using a fluorescence microscope (Figure 11) (289).

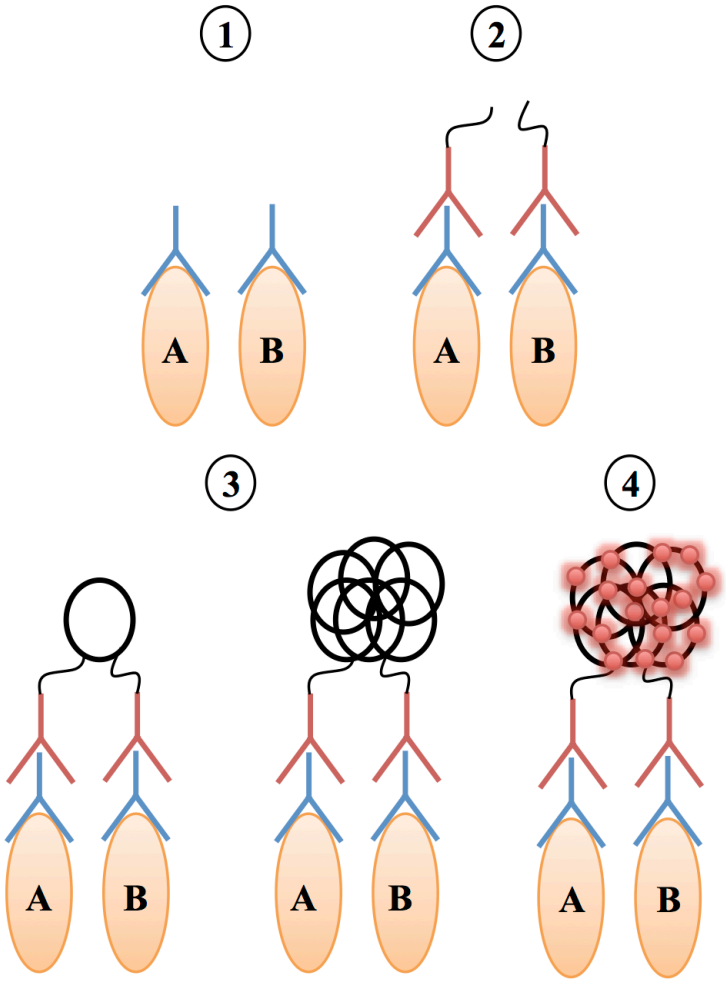


**Figure 11:** Schematic illustration of the proximity ligation assay. 1) Targeting proteins with conventional antibodies. 2) Secondary antibodies tagged with probes targets the primary antibodies. 3) Probes hybridize, which forms a circular DNA structure that then is amplified. 4) The circular DNA is with labeled with fluorescently complementary oligonucleotides

# 3 AIM OF THIS THESIS

The overall aim of this thesis was to characterize the role of WRAP53β in the DDR.

Specific aims of the papers:

I. To elucidate the role of WRAP53β in the DDR
II. To gain a deeper insight into the role of WRAP53β in DDR by using PLA
III. To study the function of overexpressed WRAP53β in carcinogenesis

# 4 RESULTS AND DISCUSSION

## 4.1 PAPER I – THE SCAFFOLD PROTEIN WRAP53β ORCHESTRATES THE UBIQUITIN RESPONSE CRITICAL FOR DNA DOUBLE-STRAND BREAK REPAIR

Several independent studies have implicated WRAP53β in the DDR. A large-scale proteomic analysis of ATM and ATR substrates in response to DNA damage revealed that WRAP53β is such a substrate (135). A genome-wide siRNA screen for identifying new genes involved in genome stabilization by monitoring γH2AX formation revealed the involvement of WRAP53β in preserving genomic stability (290). Moreover, in another genome-wide siRNA screen designed to identify factors involved in the HR repair pathway using the I-SceI endonucleus GFP-based reporter assay, WRAP53β was found to be an important factor for HR (291, 292). Furthermore, inherited mutations in WRAP53β cause the cancer predisposition disorder dyskeratosis congenita and altered expression of WRAP53β or SNPs in this gene have been associated with an increased risk of sporadic tumors such as breast and ovarian cancer, radioresistant head and neck cancer and altered DNA repair in patients with benzene-induced hematotoxicity (93, 101-103, 293, 294).

To explore the involvement of WRAP53βs in DDR, we initially used LMI to elucidate whether WRAP53βs is recruited to DNA breaks. WRAP53β accumulated at DNA lesions within minutes after LMI, indicating that WRAP53βs is high upstream in the DDR cascade. Although many WRAP53β antibodies showed localization of this protein at LMI-induced stripes, only one revealed IRIF of WRAP53β, which clearly overlapped γH2AX foci and had similar IRIF kinetics as γH2AX over a 24 h-period. To investigate how WRAP53β accumulates at DSB, we individually inhibited the three kinases in the PIKKs family: ATM, ATR and DNA-PK. ATM inhibition significantly impaired WRAP53β recruitment to DNA lesions, while ATR inhibition partially abrogated this recruitment. DNA-PKcs inhibition did not have any effect. This indicates that the recruitment of WRAP53β to DNA breaks is ATM/ATR dependent. Additionally, the recruitment of WRAP53β was impaired by knockdown of H2AX and MDC1. To study the spatiotemporal dynamics of WRAP53β, we used the I-PpoI homing endonuclease to induce DSBs at defined sites (295). It is noteworthy that WRAP53β accumulated not only at the break site but also in the surrounding region, up to 6 kb away from the damage site.

As the recruitment of WRAP53β has been established to be dependent on ATM/ATR/γH2AX/MDC1, we set out to pinpoint its localization in the DDR cascade. We knocked down WRAP53β and studied the formation of IRIF of several DDR factors. The

IRIF formation of the repair proteins 53BP1, BRCA1 and RAD51 were impaired, while γH2AX and MDC1 were unaffected. Furthermore, the E3 ligase RNF8 and RNF168, which are responsible for the repair factors to be recruited in an ubiquitylation-dependent manner, showed impaired in IRIF formation upon WRAP53β knockdown. In addition, the IRIF formation of conjugated ubiquitin at DNA breaks by RNF8 and RNF168, and the levels of ubiquitinylated H2AX were reduced in cells lacking WRAP53β.

Since WRAP53β is required for recruitment of RNF8 to DNA damage sites and downstream ubiquitylation, but does not interfere with accumulation of the upstream MDC1 protein, it appears like WRAP53β is acting at a step between MDC1 and RNF8. Therefore, we set out to determine the biochemical function of WRAP53β in RNF8 and MDC1 complex formation. We discovered that WRAP53β interacts with both RNF8 and MDC1 using co-immunoprecipitation and these interactions were enhanced upon irradiation. To further pinpoint the exact interaction sites between the proteins, we generated deletion constructs of MDC1, RNF8 and WRAP53β. This showed that WRAP53β interacts with the FHA domains of both RNF8 and MDC1. Since MDC1 also interacts with the FHA domain of RNF8, we further explored what part of this domain that is involved in the interactions with MDC1 and WRAP53β, revealing that WRAP53β interacts with amino acids 1-38, while MDC1 interacts within amino acids 39-140 of the RNF8 FHA domain.

Using deletion constructs of WRAP53β, we found that both MDC1 and RNF8 bound to the WD40 domain of WRAP53β. By dividing the WD40 domain into different combinations of repeats, we could detect that both RNF8 and MDC1 preferably interacted with repeats 2 and 3.

We next set out to study whether WRAP53β regulates the complex formation between MDC1 and RNF8 by examining the organization of interaction between WRAP53β, γH2AX, MDC1 and RNF8. By knocking down each factor separately, followed by immunoprecipitation of WRAP53β we found that the interaction between WRAP53β and MDC1 is independent of RNF8 and H2AX, and that the interaction between WRAP53β and RNF8 is independent of MDC1 and H2AX. In contrast, the interaction between RNF8 and MDC1 is dependent on WRAP53β. Since ATM phosphorylation of MDC1 is known to be required for MDC1-RNF8 interaction, we examined whether WRAP53β influences this process. However, phosphorylation of MDC1 by ATM occurred independent of WRAP53β, indicating that WRAP53β does not mediate this process. Instead, it appears like WRAP53β by simultaneous binding of MDC1 and RNF8 via its WD40 domain bring these proteins in close proximity, thus facilitating the direct interaction between MDC1 and RNF8.

To further elucidate the significance of WRAP53β for DSB repair, we knocked down this protein and analyzed clearance of γH2AX foci after irradiation. Indeed, a significant amount of γH2AX IRIF was still present 24 hours post irradiation in cells lacking WRAP53β, indicating that DSBs are left unrepaired in the absence of WRAP53β. As mentioned above, DSBs are repaired by NHEJ and HR and to study WRAP53β's involvement in these repair pathways, the I-SceI endonucleus based GFP reporter assay for HR and NHEJ repair efficiency was used. Knockdown of WRAP53β severely affected the efficiency of both HR and NHEJ repair. Additionally, in the absence of WRAP53β, irradiated cells showed a delayed recovery from G2/M arrest due to deficient DNA repair.

Furthermore, non-irradiated cells developed spontaneous γH2AX IRIF when WRAP53β was knocked down and accumulated more DSBs when analyzed using the comet assay, demonstrating that WRAP53β is important for genomic stability.

In this study, we have demonstrated that WRAP53β is a novel regulator of DSB repair by scaffolding RNF8 and MDC1 interaction, which enable ubiquitylation of damaged chromatin and facilitates downstream recruitment of necessary factors.

Nonetheless, several questions remain to be answered. For example, how can both WRAP53β and MDC1 bind the same FHA domain of RNF8? RNF8 interacts with a chromatin helicase DNA-binding protein 4 (CHD4), which is the catalytic subunit of the nucleosome remodeling and histone deacetylation (NuRD) complex. This is important for chromatin decondensation at DSBs. CHD4 is recruited to DNA lesions in a non-catalytic fashion through a phospho-independent function of the RNF8 FHA domain and mediates decondensation, which is important for the recruitment of repair factors (296). Are these proteins binding different parts of the FHA domain or do they compete for binding? There are two different predictions for the size of the RNF8 FHA domain: amino acids 13-140 or amino acids 17-111 and amino acids 130-140 has been shown to form an α helical extension (196, 197, 296). We found that an internal deletion construct of RNF8 lacking amino acids 39-109 of the FHA domain only disrupted binding to MDC1, while the binding to WRAP53β was intact. With this knowledge, we conclude that at least MDC1 and WRAP53β interact within different amino acids of the RNF8 FHA domain. Possibly, CHD4 is binding between WRAP53β and MDC1, but unfortunately, we have not investigated this matter yet.

RNF8 mediates both K48- and K63-linked ubiquitin chains depending on its interaction with the responsible E2 conjugating enzyme. The FK2 antibody used for detecting conjugating ubiquitin showed a severe decreased accumulation of ubiquitylation at DNA damage sites

when WRAP53β was knocked down. This FK2 antibody detects K29-, K48- and K63-linked mono and polyubiquitin chains and the loss of FK2 foci indicates that WRAP53β is important for both K48 and K63 ubiquitylation by RNF8. In accordance, 53BP1 recruitment, known to be dependent on both K48- and K63-linked ubiquitin chains, was lost when WRAP53β was knocked down.

WRAP53β is a multifunctional protein acting in several different cellular compartments: in the cytoplasm, Cajal bodies and at telomeres. It is possible that different posttranslational modification of WRAP53β targets this protein to the right compartment. Indeed, the ATM/ATR substrate screening study showed that WRAP53β is a substrate and we have confirmed this phosphorylation site of WRAP53β at serine 64 (data not shown).

## 4.2 PAPER II – THE PROXIMITY LIGATION ASSAY REVEALS THAT AT DNA DOUBLE-STRAND BREAKS WRAP53β ASSOCIATES WITH γH2AX AND CONTROLS INTERACTIONS BETWEEN RNF8 AND MDC1

Several DDR-related proteins especially those involved in NHEJ cannot form detectable IRIF (264). In paper II we introduce PLA in the DDR field to monitor repair proteins and obtained deeper insight of WRAP53β's involvement in the DDR cascade.

To assess whether the PLA method is applicable in the DDR field, we used this method to detect γH2AX and MDC1 interaction upon irradiation. Interestingly, several PLA signals were detected upon irradiation in a very similar pattern as the foci formation of these proteins, while no signals were acquired in non-irradiated cells. The method is both specific and sensitive, since the number of signals was reduced upon knockdown of H2AX or MDC1 or ATM inhibition. Similar results were obtained for the 53BP and γH2AX, and in combination with LMI, PLA signals could be detected along the laser stripes.

Since, the PLA method clearly could detect proteins at DNA lesions, we set out to challenge the method even further by studying WRAP53β association with γH2AX using a WRAP53β antibody that does not detect IRIF of this protein. Interestingly, PLA signals were detectable upon irradiation, confirming the findings from paper I that WRAP53β accumulates at DNA lesions and moreover, that WRAP53β associates with γH2AX. In addition, the WRAP53β-γH2AX association was detected at LMI induced stripes.

To confirm the specificity of the WRAP53β- γH2AX association, we immunoprecipitated WRAP53β and γH2AX separately and indeed, they interacted with each other in two different cell lines and the interaction was enhanced upon irradiation. By using different deletion and mutation constructs, we mapped their interaction. γH2AX did not interact with

WRAP53β deletion constructs containing only the N-terminus, the WD40 domain or the C-terminus. Neither did it bind ΔN149 or ΔC93 variants of WRAP53β, or the DC missense mutants of WRAP53β (F164L, H376Y, R398W, G435R). However, γH2AX did bind WRAP53β protein lacking 15 amino acids in its C-terminus (ΔC15). This indicates that the interaction with γH2AX requires wild type WRAP53β and proper TRiC-dependent folding of WRAP53β. Moreover, the WRAP53β-γH2AX association was ATM and ATR dependent, in line with the requirement of ATM/ATR for accumulation of WRAP53β at DSBs (paper I).

Using PLA, we also confirmed our data in paper I regarding the regulation of RNF8 and MDC1 interaction by WRAP53β. The endogenous RNF8 protein does not form visible IRIF and cannot be detected by immunoprecipitation and for that reason we used overexpressed RNF8, which forms foci and co-immunoprecipitates with WRAP53β, in paper I. In paper II, we could visualize the association between WRAP53β and endogenous RNF8 in irradiated cells using PLA. The same method also showed association between WRAP53β and MDC1 in irradiated cells. Using PLA, we also confirmed that the association between MDC1 and RNF8 was lost in WRAP53β deficient cells or upon ATM inhibition and that the phosphorylation of MDC1 appeared intact in cells lacking WRAP53β, since the MDC1-pATM association was unaffected by WRAP53β knockdown.

Our finding that the interaction between WRAP53β and γH2AX is independent of MDC1, while the foci formation of WRAP53β depends on MDC1, indicates that WRAP53β may be recruited to DSBs by two independent mechanisms. Indeed, WRAP53β accumulates both at the actual cut site of a DSB, which is typical for NHEJ factors and a region often lacking γH2AX, as well as in in the surrounding region of the breaks, which is characteristic for HR proteins and a region containing γH2AX (paper I). The retention time of WRAP53β at the actual break site was furthermore longer compared to the binding of WRAP53β at the more distal regions. This indicates that these different pools of WRAP53β are regulated differently, however the underlying mechanism(s) and whether WRAP53β has additional roles at the break site remains to be elucidated.

### 4.3 PAPER III – OVEREXPRESSION OF THE SCAFFOLD WD40 WRAP53β ENHANCES REPAIR AND CELL SURVIVAL FROM DNA DOUBLE-STRAND BREAKS

Tumors exhibiting altered responses to DNA damage and are affected very differently by genotoxic drugs, being either hypersensitive or resistant. In most cases, inactivation of DDR factors results in radiosensitivity of the tumor due to loss of DNA repair (297). Indeed, altered expression of WRAP53β is linked to both carcinogenesis and radioresistance (93, 95-

97). Here, we examined the potential influence of WRAP53β overexpression on the DNA damage response and sensitivity to DNA damaging agents.

In cells that stably overexpress WRAP53β, we could detect that WRAP53β is overexpressed both in the cytoplasm and nucleus using immunofluorescence. Chromatin fractionation further showed that the majority of overexpressed WRAP53β is in soluble form and only slightly elevated in the chromatin fraction. This finding may explain why Cajal bodies are collapsed in cells overexpressing WRAP53β, while the function of this protein in DNA repair is intact.

Interestingly, we found that overexpression of WRAP53β enhanced DSB repair using several methods. First, the clearance of γH2AX foci was faster in cells overexpressing WRAP53β compared to cells with endogenous levels of WRAP53β. Second, comet assay revealed fewer DNA breaks after irradiation in WRAP53β overexpressing cells, in accordance with faster repair. Finally, the repair efficiency of both HR and NHEJ repair was enhanced in cells overexpressing WRAP53β.

We could also link the increased efficiency in DNA repair to RNF8 in cells overexpressing WRAP53β, since these cells showed enhanced RNF8-mediated ubiquitylation of H2AX and overexpression of RNF8 itself elevated NHEJ and HR repair to a similar extent as overexpression of WRAP53β. We propose that overexpression of WRAP53β may increase the level of RNF8 in repair foci by tethering it to damaged chromatin, thus slowing down its otherwise rapid turnover and promoting ubiquitylation.

Furthermore, WRAP53β overexpressing cells are resistant to irradiation and several other DNA damaging agents, possibly due to more rapid repair, which raises the possibility that upregulation of WRAP53β may contribute to genomic stability in and survival of cancer cells.

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